UNIVERSITY OF CALIFORNIA,
IRVINE

The piRNA Pathway and Transposon Control in the Malaria Mosquito *Anopheles stephensi*

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Molecular Biology and Biochemistry

by

Vanessa Michelle Macias

Dissertation Committee:
Distinguished Professor Anthony A. James, Chair
Associate Professor Naomi Morrisette
Assistant Professor Melissa Lodoen

2015

Portions of Chapter 2 © 2014 Vanessa Michelle Macias *et al*. *Insect Molecular Biology* published by John Wiley & Sons Ltd on behalf of The Royal Entomological Society.
All other materials © 2015 Vanessa Michelle Macias

DEDICATION

"I think of you as my brother, although that sounds dumb.
These words are futile devices"

Sufjan Stevens

This work is dedicated in its entirety to George Macias.
For everyone he is a talented guitarist, a persuasive lyricist, from his beautiful mind bringing beauty and goodness into the world.

To our children he is a caring nurturer.
You bolster their growing minds with joy and affection. You feeding their curiosity and creativity by engaging with them and helping them explore their new world. You help their bodies grow in health by feeding them, wiping their butts, sponging them off when they are febrile, taking them to doctors, dentists, the park.

I cannot think of an apt descriptor of what you are to me.
You are a partner so integral to all I do and all that I am that I find hard to distinguish from yourself from myself. Should there be a second author listed on this document it would be yours, because the work that you put in for me to be able to function as an effective scientist to produce this work warrants as much credit as doing and reporting this work. You've dropped everything when I'm overwhelmed with what I'm doing to carry more of the weight so that I can move forward in confidence.
You make me believe good things about myself.
I almost can't believe that in addition to all this, I get to love being around you.
You have given me a remarkable gift: I enjoyed doing my PhD. I thrived.

Thank you mi Amor. I love you.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	v
LIST OF TABLES	vi
ACKNOWLEDGMENTS	vii
CURRICULUM VITAE	ix
ABSTRACT OF THE DISSERTATION	xii
CHAPTER 1: Genetic Engineering of Mosquitoes for Malaria Control	1
CHAPTER 2: The piRNA Pathway genes in *Anopheles stephensi*	12
Results and Discussion	13
Gene, transcript and putative protein structures of *AsAgo3 AsAug and AsPiwi*	13
Stage- and Tissue-Specific Transcript Abundance	19
Methods	23
CHAPTER 3: Characterization of Mobility of a Synthetic Autonomous Transposon	26
Results and Discussion	31
Isolation of 44C *φC31* integrase-mediated insertion genotype	31
Identification and characterization of a remobilization event	32
Methods	38
CHAPTER 4: The Impact of Stress on the piRNA Pathway and Transposon Mobility	42
Results and Discussion	45
hsp90 expression during development	45
The impact of *in-utero* heat stress on Piwi gene product abundance in embryos	45
Heat and Cold stress in Adults	49
Stress and Transposon Activity	52
The impact of oxidative stress on Piwi gene expression in adult female tissues and ovaries	55
Methods	59
CHAPTER 5: Perspectives and Future Directions	60
REFERENCES	66
APPENDIX 1 p-values From qPCR Statistical Analyses	76

TABLE OF CONTENTS

Page

APPENDIX 2 Primers 82

LIST OF FIGURES

		Page
Figure 1	Malaria Eradication Milestones	3
Figure 2	Gene structure comparisons and phylogenetic relationships of the *An. stephensi* Piwi family genes.	15
Figure 3	Abundance profiles of *AsAgo3, Aub* and *Piwi* transcripts during development	19
Figure 4	Spatial localization of *AsAgo3, Aub* and *Piwi* transcripts in ovaries	22
Figure 5	Endogenous transposase-mediated remobilization	27
Figure 6	Generation and characterization of 3.8nanos-attP44C-VM, a transgenic *An. stephensi* dual-reporter for transposon mobilization	30
Figure 7	Schematic representations of gene structures and expected amplification products resulting from autonomous mobility of transgenes	33
Figure 8	Screening for exceptional phenotypes by fluorescence microscopy	35
Figure 9	An exceptional replicate	43
Figure 10	*hsp90* expression over development	46
Figure 11	Induction of heat-shock protein and piwi expression in embryos following in-utero heat shock	48
Figure 12	Piwi gene and *hsp90* expression whole adult females	50
Figure 13	Piwi gene and *hsp90* expression in ovaries and carcass following heat-shock in blood-fed females	53
Figure 14	Piwi and *hsp90* expression following oxidative stress	58
Figure 15	Schematic representation of donor plasmid design for integration of fluorescent markers into Piwi gene open reading frames	65

LIST OF TABLES

		Page
Table 1	Piwi Identification Codes	17
Table 2	Transposons identified with homology to transcripts present in 4 hr post blood meal *An. stephensi* females	54
Table 3	Phenotypes of offspring produced from heat-treated *An. stephensi.*	56

ACKNOWLEDGMENTS

To my Father, whose brilliant mind I get to explore every day in my work. 200,000 years of human inquisition and there is still infinitely more to find, to describe, to understand. Thank you for curiosity and for giving me the endless medium for indulging it. May the beauty that I produce in my work be as pleasing to you as your beauty is to me.

Abigail, thank you for being my sounding board. I've heard over and over that if I couldn't explain it to a seven-year-old, I didn't understand it. Luckily for me, I have a beautiful companion that also happens to be a very curious seven-year-old (three when I started and eight now!). Thank you for asking questions about everything. You are brilliant and I can't wait to see more of the good and beautiful things you contribute to the world.

Amy Jude, thank you for your uninhibited laughter and for crawling into bed with me every morning. These things kept me sane and grounded through the entirety of these five years. You always reminded me what was more important than school. I found out that I was growing you when you decided that the night before my first final of my program here at UCI, I would not study or even sleep. No. I would vomit. All. Night. And then, two days before my preliminary examination, you would trick me into thinking that you were ready to be delivered and give me contractions five minutes apart for two days, until I passed my exam. You were with me all year and it was a gift.

Luna, thank you for existing. You brought a stability to our family that, honestly, I didn't think a third child could.

Megan, thank you for doing your Ph.D. at the same time as me; I know you did it so that we could commiserate on the pains of disserting. Thank you for Skype coffee dates. You use language so powerfully, and have wielded it to uplift me throughout our friendship. We have seen and heard far too little from each other during this time, but your support was constant. Also, the luxurious gifts helped! We are partners, sisters, BFFFs. I love you!

Thank you to my extensive family, Pamela, Mom, Robert, Nathanael, Dad, Dr. Mercer, Kathy, Harry, Maria, and Al for all the ways you have loved me and my little family from far away.

Tony, Sensei, thank for taking me on as a student when you didn't really want another student and when I was seven-months pregnant! You are always willing to stop what you are doing for any of us. Thank you for modelling good leadership, diplomacy, and commitment to enjoying science and life outside of science. I will be a better leader and scientist for it. You said in my first year, "grad-school should be fun." And it was, because even during the most frustrating times in lab, you supported me with discussion and encouragement. And when the results were exciting, you were just as excited as I was.

Aniko Fazekas, the mosquito whisperer. Thank you for the countless times that you seemed to manifest mosquitoes for my experiments out of thin air, for all the help that you gave me with mosquito husbandry over the years and for taking such good care in the insectary. Thank you for always sharing your wisdom with me. I'm a better scientist and parent for it.

Nijole Jasinkiene, thank you for being so willing to share your expertise with me and helping me over through protocols when something wasn't going well at the bench.

Rebeca, Chamaca, Vieja. Tú eres mi maestra y mi amiga. You have shared so much of your practical wisdom with me and I love when we work together. Every time I came to you to talk about difficultly in lab or in life, I always left with a clearer perspective and peace.

Osvaldo Marinotti, I hope that when I'm a grown up scientist that I can be like you. You're advice to me was always preceded by "you know, I was thinking about your project…" and I can't thank you enough for thinking about my projects. Your thoughts were always a marriage between seasoned wisdom and curiosity about everything.

Thank you Bianca Burini Kojin for your loyal friendship and support and for always making me laugh in lab!

Thank you William Augustine Dunn III for providing your data, time and expertise to generate the information presented in Chapter 4 on transposon transcripts expressed in *Anopheles stephensi* midguts. All samples were collected and analyzed completely by Gus and he performed the *in silico* analysis using the data collected from these samples to probe for transposon transcripts. Thank you even more, though, for your friendship and for introducing me to your incredible wife. Sarah and Gus, you are both wonderful human beings and our family is better for knowing you!

Judy Coleman, your contribution to this work is tremendous. Besides that you are an excellent lab manager and that you fill in everywhere that someone needs help, you gave weeks of work doing the steps in the *in situ* hybridization protocol that called for hazardous chemicals during my pregnancy and so helped me produce the excellent pictures in Figure 4. And to boot, you were adamant that you were enjoying it!

Alyssa Olguin Jimenez, thank you for including us in your extensive family, since I have been away from my family for the years I've worked on this dissertation.

Thank you Mariangela Bonizonni for your profound insight during lab meeting discussions of my project and for helping me with the phylogenetic analysis presented in Figure 2.

Thanks to Dr. Igor Sharakov at Virginia tech for many helpful discussions about piRNAs and for helping me locate the chromosomal locations of new insertions on in the 3.8nanos-attP-44C-VM line.

Thank you for all the people who've care for my children these five years, you are too numerous to name here. But Marie Tsasaan, you deserve special mention. You have been like a part of our family, our children running in and out of each other's homes as if they were their own. You even had the courage to take on our three while George joined me at a week-long conference; just to remind you, that's five children to one super-powered parent. Thank you, we love you.

Thanks to Naomi Morrissette and Melissa Lodoen for your guidance and helpful insights into my project.

Chapter 2 of this thesis/dissertation is a reprint of the material as it appears in "piRNA pathway gene expression in the malaria vector mosquito Anopheles stephensi." Which was published in *Insect Molecular Biology in 2014*. Thank you to Wiley Publishing for the freedom to reproduce sections and figures from Macias *et al.* 2014 freely in this document. Thank you to co-authors Judy Coleman and Mariangela Bonizonni for your significant contributions to this work and co-author Anthony James who supervised this work.

CURRICULUM VITAE

Vanessa Macias 3222 Verano Place, Irvine, CA 92617 575-621-9704 maciasv@uci.edu

Education

Ph.D. Candidate and Graduate Student Researcher

University of California, Irvine

Department of Molecular Biology and Biochemistry

Advisor: Dr. Anthony A. James, Distinguished Professor

Advancement date: October 31, 2012

Masters Degree in Microbiology
New Mexico State University, 2010
Thesis:: "Target of Rapamycin signaling and fecundity in the malaria mosquito *Anopheles gambiae*"
Course work including: Disease Vector Biology, Parasitology, Statistics for Biological Sciences, Molecular Cell Biology, Genomic technologies.

Bachelor of Science Degree in Microbiology
New Mexico State University, 2007
Minors in Chemistry and Biochemistry; graduated a Crimson Scholar with honors, 3.8 GPA
Course work including: Biochemistry, Microbiology, Microbial Genetics, Emerging Infectious Diseases, Virology, Anatomy and Physiology, Immunology, Organic Chemistry, Physical Chemistry, Intermediary Metabolism, Genetics.

Publications

Impact of genetic modification of vector populations on the malaria eradication agenda, 2015
In: Genetic Control of Malaria and Dengue
Vanessa Macias and Anthony A. James

The piRNA pathway and transposon control in *Anopheles stephensi,* 2014
Vanessa Macias, Judy Coleman, Mariangela Bonizzoni, Anthony James
Insect Molecular Biology

Posters and Presentations

World Malaria Day 2015 Bay Area Symposium
Session Chair
Presentation and Poster: "Mosquitoes that block malaria parasite transmission: Soundbites on the road from lab to field"

Posters and Presentations (continued)

Annual Meeting of the American Society of Tropical Medicine & Hygiene, 2014
Presentation: "The piRNA Pathway and Stress"

University of California 1st Annual Associate Graduate Student Symposium, 2014
Presentation: 'Crafting a technology that will never fly: the piRNA pathway and transposon control in *Anopheles stephensi*"

Orange County Graduate Women in Science Sigma Chapter 25th Spring Science Conference, 2014
Presentation: "The piRNA pathway and transposon control in *Anopheles stephensi*"

SOVE 6th International Congress, 2013
Poster: "Piwi gene expression characterization in the human malaria vector *Anopheles stephensi*"

New Mexico State University Graduate Research and Arts Symposium, 2010
Presentation: "Target of Rapamycin signaling and fecundity in the malaria mosquito *Anopheles gambiae.*"

New Mexico State University Biosymposium, 2010
Poster: "Target of Rapamycin signaling and fecundity in the malaria mosquito *Anopheles gambiae.*"

Volunteer and Leadership Activities

Guest Lecture for Chapman University Biology 300-General Genetics, 2014 and 2015
"On Mosquitoes, Transposons and piRNAs: Little players with powerful genetic capabilities!"

Irvine Unified School District Science Fair, 2015
Judged 7th and 8th grade participant on experiment design, creativity and execution of original science projects.

Irvine Unified School District "Ask a Scientist" Night, 2014
Consulted with middle school students on experimental design in preparation for science fair projects.

Fellowships

6th International Congress of the Society for Vector Ecology Travel Award, 2013

Recipient of New Mexico State University Biology Department Tuition Fellowship, 2008

Affiliations

American Society of Tropical Medicine & Hygiene

Graduate Women in Science

American Association for the Advancement of Science

Society for Vector Ecology

Teaching Assistantships

Molecular Cell Biology
Summer 2015
Biosciences Department, Univ. California, Irvine

Biochemistry
Summer 2015
Biosciences Department, Univ. California, Irvine

Microbiology Laboratory and Discussion Section,
Winter 2012, Winter 2014
Molecular Biology and Biochemistry Department, Univ. California, Irvine

Microbial Genetics
Winter 2012, Winter 2014
Biosciences Department, Univ. California, Irvine

Human Anatomy
Spring 2008-Spring 2010
Biology Department, New Mexico State University

Employment History

Respite Care Provider
Dec 2005-Jan 2008
Tresco Inc., Las Cruces, NM

Dance Instructor
Sept 2003-Dec 2006
Hillrise Elementary School, Las Cruces, NM

Medical Assistant and Lab Technician
Jun 2001-Jan 2008
Dr. Ann S Mercer, M.D. PA Office of Internal Medicine, Las Cruces, NM

ABSTRACT OF THE DISSERTATION

The piRNA Pathway and Transposon Control in the Malaria Mosquito *Anopheles stephensi*

By

Vanessa Michelle Macias

Doctor of Philosophy in Molecular Biology and Biochemistry

University of California, Irvine, 2015

Distinguished Professor Anthony A. James, Chair

The ability of transposons to mobilize to new places in a genome enables them to introgress rapidly into populations. Transposons have been used as tools in mosquitoes to genetically transform a number of species including *Anopheles stephensi*, a vector of human malaria. These mobile genetic elements also have been proposed as tools to drive anti-pathogen effector genes into wild mosquito populations to replace pathogen-susceptible insects with those engineered genetically to be resistant to or unable to transmit a pathogen. The piRNA pathway has been characterized recently in the germ-line of the fruit fly, *Drosophila melanogaster*, and is responsible for down-regulating transposon mobility. In order to use transposons effectively for vector and disease control, we need an understanding of the biology of the interplay between mosquitoes and synthetic transposon constructs. Presented here are: 1) evidence that components of the piRNA pathway are present in *An. stephensi* and expressed in a manner spatially and temporally appropriate for transposon control; 2) a proof-of-principle for a synthetic autonomous transposon-based construct, showing that exogenous genes can be encoded to self-mobilize; and 3) an investigation into a link between stress, transposon mobility and the piRNA pathway.

Chapter 1

Genetic Engineering of Mosquitoes for Malaria Control

Reports of progress on the global malaria situation are a mixed. The World Health Organization (WHO) has evidence for a continuing reduction in mortality, attributed in part to use of bed nets and combination drug therapies (White *et al*., 2014; WHO, 2013). Some 3.3 million lives are estimated to have been saved since 2001. This success supports efforts to increase implementation of existing control measures with the expectation that they will continue to lower malaria incidence. However, these hard-won gains are threatened by inadequate public health infrastructures, the increasing scale over which previously-successful programs must be applied, and insecticide and parasite drug resistance (Cohen *et al*., 2012; WHO, 2013). Furthermore, a number of recently-recognized challenges have been identified that add to an already complex situation. These include the impact of global warming on mosquito vector distribution and the emergence of additional species of malaria parasites that can infect humans (Antinori *et al.*, 2013; Calderaro *et al*., 2013; Sheffield & Landrigan, 2011). Thus, while there is much to celebrate about the recent reductions, we must continue to apply proven technologies while at the same time develop new disease-control tools.

The renewed call for malaria eradication stimulated cooperative planning among the malaria public health and research communities to develop agendas for reaching this goal (The malERA Consultative Group on Integration Strategies, 2011). Eradication was defined in the agenda as the reduction of transmission below a threshold level that achieves an impact on the basic reproductive rate (R_o) of the disease such that $R_o < 1$. However, it is

more straightforward to express it as the complete absence of parasites in humans so that they are not able to infect mosquito vectors, and the complete absence of parasites in mosquitoes so that they cannot infect humans. Recent infections of humans by parasites found previously only in non-human primates requires addressing sources of infections that originate in animal reservoirs (Antinori *et al*., 2013; Calderaro *et al*., 2013).

Eradication is achieved through the phased operational targets of control, pre-elimination, elimination and prevention of reintroduction (WHO, 2014) (Figure 1). The WHO defines control as <5% positive slides in all patients presenting with fever and elimination as no cases of locally-acquired malaria for a period of three years as a result of deliberate control efforts. Eradication is the global elimination of malaria. This is an ambitious goal and there is a consensus that it is unlikely that any single technology will be sufficient to achieve it (The malERA Consultative Group on Integration Strategies, 2011). Contributions are needed from diagnostic, therapeutic and prevention domains, and the knowledge from a broad array of scientific disciplines must be recruited to support this effort.

It is important to ask how the goal of eradication informs the research agenda in the many contributing disciplines. This question put explicitly to vector biologists was answered with the elaboration of a number of critical needs (The malERA Consultative Group on Vector Control, 2011). Existing broadly-applicable (insecticide treated nets, indoor residual sprays) and region-specific (environmental modification) vector-targeted prevention tools were sufficient to achieve control

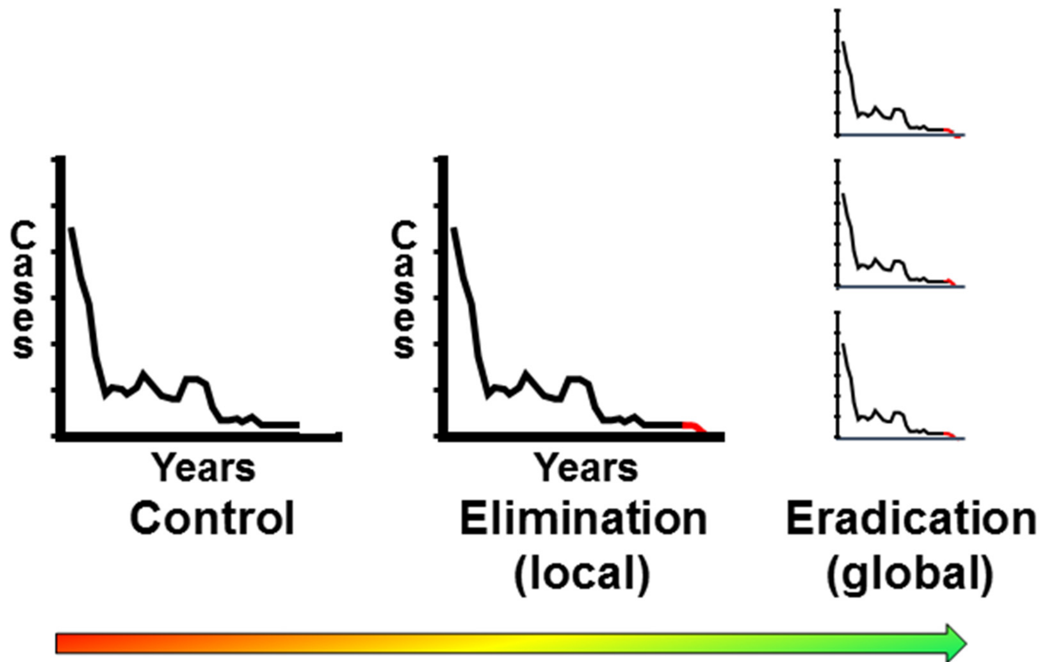


Figure 1. Malaria eradication milestones. Malaria eradication (right) will be achieved through a series of phases that progress (arrow) from control (left) through elimination (center). The x- and y-axes show numbers of cases and years, respectively, in arbitrary units. The red portion of the curve represents achieving elimination.

and elimination in many regions of the world. It is essential to use these tools where feasible and efficacious. However, there are malaria endemic areas where it has not been possible to achieve control and elimination. This can be due to the failure to apply the currently-available tools because of geographical, political and economic difficulties, circumstances where these tools were applied but did not work (for example, insecticide-treated nets do not impede out-door, day-time feeding mosquitoes), and those situations where the tools worked previously but are no longer effective (for example, the emergence of insecticide resistance) or can no longer be delivered because of failed or overwhelmed public health infrastructure. Thus, there is a clear need in the vector biology contributions to malaria eradication for better use of existing control tools and the development of novels ones to complement them.

Two of the major challenges to malaria eradication are the heterogeneity/complexity of transmission dynamics and difficulties in sustaining control efforts (Cotter *et al*., 2013; Luckhart, Lindsay, James, & Scott, 2010; Spielman, Kitron, & Pollack, 1993). This complexity is evident in the vector components by the large number of *Anopheles* mosquito species that have been implicated world-wide in malaria parasite transmission. There are some ~450 described *Anopheles* species, 68 of which are known to transmit human malaria, and as many as 40 are identified as major vectors (Hay et al., 2010; Kiszewski et al., 2004). Indonesia alone has as many as 24 species involved in regional parasite transmission (Elyazar *et al*., 2013). Each of these species has its own biology associated with host-preferences, feeding behavior (indoor/outdoor, day/night, *etc*.), mating

behavior, breeding-site preferences, and vector competence[1], all of which affect their vectorial capacity. It is a significant challenge to find a single tool that accommodates all of this diversity, and this supports arguments for having multiple approaches to vector control that can be applied as needed and where effective (The malERA Consultative Group on Vector Control, 2011).

Sustainability is a major challenge to all public health efforts and can be destabilized by both success and failure. Successful public health creates the 'public health paradox'; when it is working nothing is happening. Specifically, good public health practices are characterized by the lack of disease. It is difficult under these circumstances to continue to devote resources to a problem that is perceived not to exist (WHO, 2014). Withdrawal of support can lead to disease re-emergence and the ensuing costs of reasserting control are likely to be greater than those incurred by maintaining it (Tatarsky *et al*., 2011). Sustainability of vector control also has additional, intrinsic features that lead directly to failure. The most often cited is the development of insecticide resistance, and this has had a major negative impact on maintaining control in many areas of the world (Sokhna, Ndiath, & Rogier, 2013). Additionally, migration of infected humans and mosquitoes also compromises sustainability; malaria epidemics and focal outbreaks can occur in regions that have achieved elimination through the absence of the parasites but still have local competent vectors (Cohen *et al.,* 2012; Center for Disease Control and Prevention, 1986).

[1] Vector competence is a measurement of the intrinsic ability of the insect to transmit a pathogen and includes genetic components(Hardy, Houk, Kramer, & Reeves, 1983). Vectorial capacity is a measurement of the efficiency of pathogen transmission, and of which vector competence is a parameter(D. L. Smith et al., 2012).

The prospects for success in malaria eradication will depend on how well major scientific disciplines can provide tools that can address complexity and sustainability. For example, chemistry coupled with physiological insights can produce new insecticides for the vectors and prophylactic and therapeutic drugs for the parasites; these agents may have a sufficiently broad spectrum of application to be useful in managing complexity. Ecological studies can guide rational, community-wide, environmental management to remove mosquito breeding sites, and behavioral sciences can inform at-risk populations about adopting personal-protection measures (for example, bed nets and repellents), and these also could have an impact on complexity. Immunology provides tools to probe disease progression and the basis for developing vaccines, and so contributes to sustainability at the individual patient level. Importantly, new tools being developed in the field of genetics can offer sustainability at a regional level.

Genetic approaches that target mosquito vectors as a means of disease control have been in consideration since the 1940s (Klassen & Curtis, 2005). Indeed, sterile insect technologies were used to control a vector mosquito in Central America. This success was unsustainable, mostly due to civil unrest, and negative publicity in a separate effort in India decreased enthusiasm for these approaches (Jayaraman, 1997; "Oh, New Delhi; oh, Geneva. (editorial)," 1975). However, the development of powerful molecular biological tools re-kindled enthusiasm for developing genetic control strategies. Genetic manipulation of mosquitoes in previous efforts involved radio- or chemical-sterilization (efforts in these areas are still ongoing--Bellini, Medici, Puggioli, Balestrino, & Carrieri, N.D.; Damiens, Vreysen, & Gilles, 2013; Gato *et al*., 2014; Harris *et al*., 2013; Lwetoijera *et al.*, 2014;

Madakacherry, Lees, & Gilles, 2014; Maïga *et al.*, 2014; Ndo *et al.*, 2014; Oliva *et al.*, 2014, 2013; Yamada *et al*., 2015; Yamada, Vreysen, Gilles, Munhenga, & Damiens, 2014), but with the advent of mosquito transgenesis we gained the ability to genetically engineer specific phenotypes in mosquitoes. The potential to design gene constructs that could cause sterility resulting in population reduction analogous to previous sterile insect techniques or that could enable the mosquito to interrupt parasite development during its reproductive cycle in the mosquito fostered research to exploit these technologies for malaria control (WHO, 1991).

Initially, mosquito transgenesis was made possible by transposons, mobile genetic elements that enabled researchers to encode exogenous synthetic constructs into the mosquito genome (McClintock, 1987). The P element, a transposon that could be used to transform *Drosophila* species, was responsible for a revolution in genetic work in *Drosophila melanogaster*, but was essentially useless for mosquito transgenesis. The first transformations in mosquitoes were made using *mariner* and *Hermes* elements in *Aedes aegypti,* and not long after, *piggyBac* elements were used to transform *Anopheles species* (Catteruccia *et al*., 2000; Coates *et al.*1998; Grossman *et al*., 2001; James, 2005; Terenius *et al.*, 2008)*.* The development of these genetic tools enables the integration of exogenous genes into mosquito genomes, and the progress of the technology has led to the generation of mosquitoes equipped with anti-malarial effector genes that can inhibit the complete development of the parasite during its life cycle in the mosquito, such that no parasites enters the salivary glands and we therefore expect that no parasite would be transmitted to the next human on which the mosquito feeds (Isaacs *et al*., 2011, 2012).

With disease-resistant mosquitoes available and more likely to come, mechanisms to drive the genes of a transgenic mosquito into a wild-population (gene-drive mechanisms) for disease control are being explored. Using transposons and their natural ability to invade populations is an attractive option for gene drive. Some transposons spread more rapidly than generally seen for fixation of a new genotype, remarkably overcoming natural selection while conferring no fitness advantage and likely a fitness disadvantage (Engels, 1997). The P element is predicted to have been transmitted horizontally from one species of *Drosophila* to another early in the 20th century and was present in flies globally within decades (Daniels *et al.*, 1990).

Not only did the P element boost *Drosophila* research, it led to the discovery and characterization of a phenomenon called hybrid dysgenesis. The P element's species jump into *D. melanogaster* occurred, serendipitously, just after lab colonies had been established and some crosses between wild- and colony-derived individuals produced aberrant phenotypes in the resulting progeny, such as unusually high recombination and sterility. Engels relates that the mysterious nature of these data earned them dismissal as idiosyncrasies of specific strains but it was realized soon that the traits were incompatibilities between two classes of strains into which wild and laboratory populations could be divided (Engels, 1997; Kidwell, Kidwell, & Sved, 1977). It was soon discovered that these phenomena were a result of the lab stock female flies being naïve to the mobile DNA element, which had already spread through wild populations. The wild populations had gained the ability to control P elements, which if left unattended would

certainly destroy the population by genomic disruption. Females of the wild populations donated P element control to their offspring as a cytoplasm component, which laboratory females did not have. As a result, the P element was allowed to remobilize and disrupt genomic DNA in progeny from laboratory stock females and wild-type males. It was not known at the time that this control was in the form of transposon-specific RNA moieties, now called piwi RNAs (piRNAs), and this would not be known until the discovery and characterization of RNA inhibition by small RNAs.

The first two RNA inhibition pathways to be well-characterized are the microRNA (miRNA) and double-stranded RNA (dsRNA) pathways. These both process long double-stranded RNAs from different sources into precise lengths of small RNAs; siRNAs are 21 nt long and miRNA are 22 nt long (Reviewed in Carthew & Sontheimer, 2009). These small RNAs repress gene expression by recruiting RNase enzymes of the Argonaut gene family to mRNAs by complementary base-pairing. Information was slower in coming for the piRNAs; they are slightly larger (24-30 nt), with a wider range of size, bind different proteins and are expressed preferentially in the germline (Brennecke *et al*., 2007; Djikeng, Shi, Tschudi, & Ullu, 2001; Hamilton, 2002; Llave, 2002; Mette, van der Winden, Matzke, & Matzke, 2002; Reinhart & Bartel, 2002). In 2007, Brennecke *et al.* and others reported on piRNAs encoded in the *D. melanogaster* germline that led to a model of inherited transposon control whereby embryos are protected from the DNA-damaging effects of transposon movement by RNAi-mediated degradation of transposon mRNA using maternally-inherited antisense RNA (Brennecke *et al*., 2007; Pélisson, Sarot, Payen-Groschêne, & Bucheton, 2007; Saito *et al.*, 2006; Sarot, Payen-Groschêne, Bucheton, & Pélisson, 2004; Vagin *et al*., 2006).

The biology of the piRNA pathway in mosquitoes is relevant to both applied and basic aspects of disease vector research. Understanding the dynamics of transposon movement in the malaria vector, *An. stephensi*, and the control of this process is an interesting basic science question to investigate in an organism with a different reproductive strategy than *Drosophila* species (vector mosquitoes require a blood meal for development of progeny). Additionally, information on transposon control in mosquitoes is essential to proceeding intelligently with the design of gene-drive systems based on transposons. Further, it has become clear in recent work in model organisms that the piRNA pathway can be triggered to control newly-introduced gene constructs; the locus is integrated into a piRNA cluster, a locus from which the entire sequence is used to produce piRNAs (Le Thomas *et al*., 2014; Olovnikov *et al*., 2013). This has important implications for continuing work in introducing functional gene constructs, particularly at the population level. Should a transgene come under the control of the piRNA pathway, sequence elements including control elements, markers and effector genes could potentially be inhibited from further use in such a population.

The piRNA pathway is likely more broadly relevant to disease vector biology. Mosquitoes are majors vectors of viral infections to humans and animals and molecular aspects of mosquito-virus interactions are major foci of research. Recent data implicates the piRNA in virus control; viral-specific piRNAs derived from *D. melanogaster, Anopheles* and *Aedes* cell lines challenged with viral infection have been isolated and piRNAs have been identified as expressed from viral regions of mosquito vector genomes (Aguiar *et al*.,

2015; Chotkowski *et al*., 2008; Léger *et al*., 2013; Morazzani, Wiley, Murreddu, Adelman, & Myles, 2012; Schnettler *et al*., 2013; Vodovar *et al*., 2012). The piRNA pathway also may regulate gene expression since a proportion of piRNAs sequenced in *D. melanogaster* and *Ae. aegypti* are specific to endogenous protein-encoding genes (Akbari *et al*., 2013; Arensburger, Hice, Wright, Craig, & Atkinson, 2011; Brennecke *et al*., 2007).

Indeed, as in all disciplines contributing to malaria elimination and eradication, research on all levels, from basic to applied, will be necessary to hone existing tools and to craft technologies that can become new tools for executing and solidifying gains against this tenacious disease. Understanding the interplay between mosquito biology and exogenous DNA elements will inform an intelligent strategy for using transgenic mosquitoes to address the global problem of malaria. The studies reported in the following chapters represent attempts to untangle the specifics of one aspect of this interplay, specifically between transposons and the piRNA pathway, with a consideration of whether and how the use of synthetic elements as would be used in mosquito and disease control are informed by that relationship.

Chapter 2

The piRNA Pathway Genes in *Anopheles stephensi*

The piRNA pathway was proposed to play a role in transposon regulation in mosquitoes of the genus *Aedes* based on small RNA and genomic sequencing data (Akbari et al., 2013; Arensburger et al., 2011). However, some notable differences are observed between data from *Ae. aegypti* and those derived from *D. melanogaster*. The *Ae. aegypti* genome has a much higher proportion of transposon sequences (47% in *Ae. aegypti* compared to 15.8% in *D. melanogaster*) but a smaller representation of piRNAs targeting transposons (~20-40% in *Ae. aegypti* compared with 50% in *D. melanogaster*) (Akbari et al., 2013; Arensburger et al., 2011; Kaminker et al., 2002; Nene et al., 2007; C. D. Smith *et al.*, 2007). Furthermore, there are different effectors of the piRNA pathway between *D. melanogaster* and *Ae. aegypti*. The Piwi sub-family of Argonaut proteins are the primary protein players in the piRNA pathway (Höck & Meister, 2008). In *D. melanogaster* there are three Piwi family proteins that are loaded with piRNAs to target and cleave RNA for degradation; these are Piwi, Aubergine (Aub) and Argonaut 3 (Ago3) and have been shown to be primarily present in the germline (Brennecke *et al.*, 2007; Gunawardane *et al.*, 2007; Harris & Macdonald, 2001; Li *et al.*, 2009). In *Ae. aegypti* there are eight putative Piwi family genes annotated in VectorBase, one with homology to *DmAgo3* and seven that show homology to *DmPiwi* and *DmAub,* with various spatial and temporal expression profiles. Thus, the piRNA pathway may function differently in mosquitoes and fruit flies. However, data collected from the mosquito genus *Anopheles* look more similar to corresponding data in *Drosophila* than *Aedes*. For example, one-to-one orthologs exist in *An. gambiae* for each of the three *D. melanogaster Piwi* genes. Additionally, anopheline mosquitoes have a

relatively small genome resulting from lower transposon representation compared to the *Ae. aegypti* genome (Hoa *et al.* 2003; Holt et al., 2002; Kaminker et al., 2002; Marinotti et al., 2013; Nene et al., 2007; Zhou, Liao, Jia, Cheng, & Li, 2007).

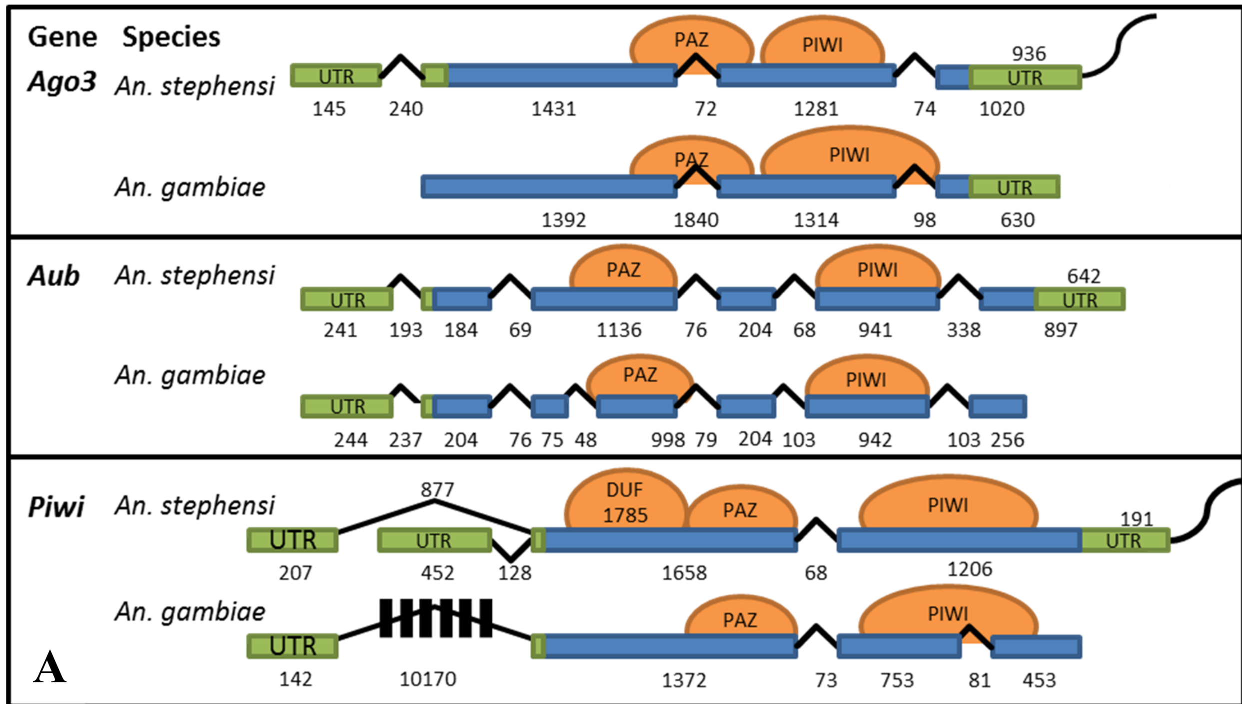
As a first step to understanding the role that the piRNA pathway plays in transposon control and the relevance of this role to genetic-engineering based technologies in *An. stephensi,* the Piwi family genes were identified and their expression profiles measured at the different developmental stages and in germline tissues of the adult female. We expected that if this pathway regulates transposon control to mitigate germline transposon activity, that these genes would be abundant in the ovaries and embryos. Our observations in *An. stephensi* support the hypothesis that the piRNA pathway plays a role in transposon control in this species; we find that *Piwi, Aub*, and *Ago3* display expression characteristics appropriate for priming an egg for exposure to paternal transposons: transcripts are detectable in the germ-line tissue of adult mosquitoes, become increasingly more abundant in the ovaries with egg development and are found in the embryos.

Results and Discussion

Gene, transcript and putative protein structures of *AsAgo3, AsAub* and *AsPiwi*.

Alignments of the *An. gambiae Ago3, Ago4 (Aub)* and *Ago5* (*Piwi)* transcript sequences with the *An. stephensi* genome (Assembly: AsteI2) and transcriptome yielded partial matches that aligned with >78% identity. According to sequence similarity, the genes ASTEI04992, ASTEI03833 and ASTEI06803 were designated *AsAgo3*, *AsAub* and *AsPiwi,* respectively (Figure 2, Table 1) and are referred to collectively as the Piwi genes throughout this

document. Genomic DNA sequences from the *An. stephensi* Indian strain (VectorBase.org) were used to design oligonucleotide primers for gene amplification studies to determine *AsAgo3*, *AsAub* and *AsPiwi* structures and the complete sequences of their corresponding transcripts. Single transcripts were identified for both *AsAgo3* and *AsAub*, while amplification of the 5'-end of *AsPiwi* revealed a novel, alternative first exon that aligned to the genome within the first intron (Figure 2A). Both of the *AsPiwi* transcripts have the same translation start site and are predicted to produce identical proteins. Transcripts of 3895, 3316, and 3071 nucleotides (nt) in length from the start of transcription to the beginning of the poly-adenosine sequences were observed for *AsAgo3,* and *AsPiwi* isoforms A and B, respectively. *AsAub* transcripts extend beyond the first polyadenylation signal in the 3'-end UTR, such that the transcript is larger than 3615 nt. Complete transcripts have been deposited in VectorBase; the transcript sequences reported here supplement previous annotations of these transcripts by providing: 3'- and 5'-end untranslated regions (UTRs) for both *AsAgo3* and *AsPiwi*, an entirely new sixth intron for *AsAub* and an alternate 5'-end UTR for *AsPiwi.* Comparisons of the *An. stephensi Ago3, Aub* and *Piwi* genes with those of *An. gambiae* annotated in VectorBase revealed a number of differences: a lack of data supporting a 5'-end UTR for *AgAgo3* and 3'-end UTR for *AgAub* and *AgPiwi*; *AgAub* encodes an additional intron compared to *AsAub; AsPiwi* does not have the large first intron (10,170 nt) found in *AgPiwi*; and as noted above, *AsPiwi* encodes an alternate first intron (Figure 2A).


Gene
Species
Ago3
An. stephensi
UTR
145
240
1431
PAZ
72
PIWI
1281
74
936
UTR
1020
An. gambiae
1392
PAZ
1840
PIWI
1314
98
UTR
630
Aub
An. stephensi
UTR
241
193
184
69
PAZ
1136
76
204
68
PIWI
941
338
642
UTR
897
An. gambiae
UTR
244
237
204
76
75
48
PAZ
998
79
204
103
PIWI
942
103
256
Piwi
An. stephensi
877
UTR
207
UTR
452
128
DUF
1785
PAZ
1658
68
PIWI
1206
191
UTR
An. gambiae
UTR
142
10170
PAZ
1372
73
PIWI
753
81
453
A

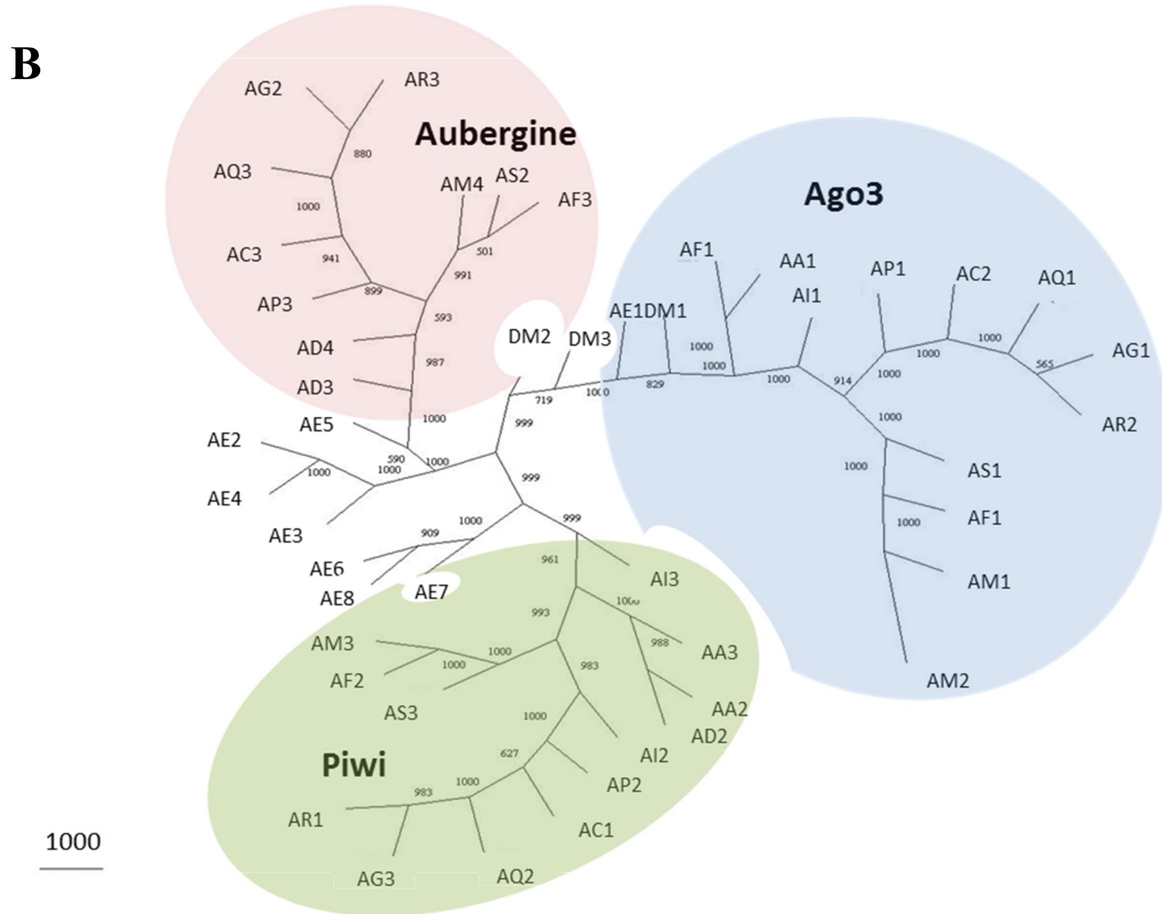

B
Aubergine
Ago3
Piwi
AG2
AR3
AQ3
AC3
AP3
AD4
AD3
AE5
AM4
AS2
AF3
AE2
AE4
AE3
AE6
AE8
AE7
DM2
DM3
AE1
DM1
AF1
AA1
AI1
AP1
AC2
AQ1
AG1
AR2
AS1
AF1
AM1
AM2
AI3
AA3
AA2
AD2
AI2
AP2
AC1
AQ2
AG3
AR1
AM3
AF2
AS3
1000

Figure 2: Gene structure comparisons and phylogenetic relationships of the *An. stephensi* Piwi family genes. A) Schematic representations of transcription products for *An. stephensi Ago3, Aub* and *Piwi* detected in samples of ovaries collected at 48 hr PBM. Exons and introns are represented by boxes and lines, respectively, with the length in nucleotides indicated below each. Untranslated regions are colored green, open-reading frames are blue, predicted protein-binding domains are represented by orange circles and the polyA tail is the curved line at each 3'-end. B) Phylogenetic tree generated from alignment of predicted amino acid sequences for Piwi family proteins in *An. stephensi* (As), *An. gambiae* (Ag), *Aedes aegypti* (Aae), and *Drosophila melanogaster* (Dm). Bootstrap values between genes are listed between each pair of corresponding nodes. Genes from other mosquito species are represented: AA, *Anopheles albimanus*; AC, *Anopheles christi*; AD, *Anopheles darlingi*; AI, *Anopheles dirus*; AF, *Anopheles funestus*; AM, *Anopheles minimus*; AP, *Anopheles epiroticus*, AQ, *Anopheles quadriannulatus* and AR, *Anopheles arabiensis*. The digits following species name designations are arbitrary and correspond to the gene names listed in Table 1.

Table 1: Piwi gene identification codes

Gene	Species	Strain	Gene ID	Tree Identifier
Ago3	*Anopheles stephensi*	Indian strain, AsteI 2.1 geneset	ASTEI04992	AsAgo3
		SDA-500 strain, AteS 1.0 geneset	ASTE009599	AsAgo3
	Anopheles gambiae	PEST, Agamp3.7 geneset	AGAP008862	AgAgo3
	Aedes aegypti	Liverpool, AaegL 1.4 geneset	AAEL007823	AaeAgo3
	Drosophila melanogaster		3355150	DmAgo3
	Anopheles albimanus	STECLA, AalbS1.1 geneset	AALB001790	AA1
	Anopheles arabiensis	Dongola, AaraD1.1 geneset	AARA009401	AR2
	Anopheles christi	ACHKN1017, AchrA1.1 geneset	ACHR007454	AC2
	Anopheles darlingi	Coari, AdarC2.2 geneset	ADAC008631	AD1
	Anopheles dirus	WRAIR2, AdirW1.1 geneset	ADIR009721	AI1
	Anopheles epiroticus	Epiroticus2, AepiE1.1 geneset	AEPI002451	AP1
	Anopheles funestus	FUMOZ, AfunF1.1 geneset	AFUN000985	AF1
	Anopheles minimus A	MINIMUS1, AminM1.1 geneset	AMIN001620	AM1
			AMIN001621	AM2
	Anopheles quadriannulatus A	SANGWE, AquaS1.1 geneset	AQUA007856 AQUA007857	AQ1
Aubergine	*Anopheles stephensi*	Indian strain, AsteI 2.1 geneset	ASTEI03833	AsAub
		SDA-500 strain, AteS 1.0 geneset	ASTE011384	AsAub
	Anopheles gambiae	PEST, Agamp3.7 geneset	AGAP009509	AgAub
	Aedes aegypti	Liverpool, AaegL 1.4 geneset	AAEL008076	AaePiwi1
			AAEL008098	AaePiwi2
			AAEL013692	AaePiwi3
			AAEL007698	AaePiwi4
	Drosophila melanogaster		34524	DmAub
	Anopheles arabiensis	Dongola, AaraD1.1 geneset	AARA011871	AR3
	Anopheles christi	ACHKN1017, AchrA1.1 geneset	ACHR004324	AC3
	Anopheles darlingi	Coari, AdarC2.2 geneset	ADAC006051	AD3
	Anopheles dirus	WRAIR2, AdirW1.1 geneset	ADIR006020	AI4
	Anopheles epiroticus	Epiroticus2, AepiE1.1 geneset	AEPI004893	AP3
	Anopheles funestus	FUMOZ, AfunF1.1 geneset	AFUN005296	AF3
	Anopheles minimus	MINIMUS1, AminM1.1 geneset	AMIN001881	AM4
	Anopheles quadriannulatus A	SANGWE, AquaS1.1 geneset	AQUA005807	AQ3
Piwi	*Anopheles stephensi*	Indian strain, AsteI 2.1 geneset	ASTEI06803	AsPiwi
		SDA-500 strain, AteS 1.0 geneset	ASTE008262	AsPiwi
	Anopheles gambiae	PEST, Agamp3.7 geneset	AGAP011204	AgPiwi
	Aedes aegypti	Liverpool, AaegL 1.4 geneset	AAEL013233	AaePiwi5
			AAEL013227	AaePiwi6
			AAEL006287	AaePiwi7
	Drosophila melanogaster		34521	DmPiwi
	Anopheles albimanus	STECLA, AalbS1.1 geneset	AALB010564	AA2
			AALB010570	AA3
	Anopheles aribiensis	Dongola, AaraD1.1 geneset	AARA000129	AR1
	Anopheles christi	ACHKN1017, AchrA1.1 geneset	ACHR009351	AC1
	Anopheles darlingi	Coari, AdarC2.2 geneset	ADAC008732	AD2
	Anopheles dirus	WRAIR2, AdirW1.1 geneset	ADIR000087	AI2
			ADIR000092	AI3
	Anopheles epiroticus	Epiroticus2, AepiE1.1 geneset	AEPI006652	AP2
	Anopheles funestus	FUMOZ, AfunF1.1 geneset	AFUN004060	AF2
	Anopheles minimus	MINIMUS1, AminM1.1 geneset	AMIN008377	AM3
	Anopheles quadriannulatus A	SANGWE, AquaS1.1 geneset	AQUA005063	AQ2

The encoded amino acid sequences were predicted from all three transcripts using the ExPASy online translate tool (http://web.expasy.org/translate). The SMART online protein domain prediction tool (http://smart.embl-heidelberg.de/) predicts both PAZ and PIWI domains (Höck & Meister, 2008) (Figure 2A). Additionally the *AsPiwi* predicted proteins have a conserved domain of unknown function (DUF 1785) present in Argonauts and co-occurring with PIWI domains (Kurscheid et al., 2009; Poulsen, Vaucheret, & Brodersen, 2013; Su, Zhu, Wang, & Jang, 2009; Zheng, 2013). Predicted amino acid sequence alignments show a high percent identity between *An. stephensi Ago3, Aub* and *Piwi* and putative orthologous proteins in *D. melanogaster* (48%, 49% and 44%, respectively) and *An. gambiae* (77%, 89% and 72%, respectively), supporting the conclusion that the corresponding genes represent orthologs (Figure 2B). Interestingly, AsAub and AsPiwi were more similar to each other than to either DmAub or DmPiwi, as reported previously for these proteins in *An. gambiae* (Hoa et al., 2003). A survey of other available anopheline genomes (VectorBase) results in the identification of one orthologous gene corresponding to each of the three Piwi proteins in all species with a few exceptions. Three Piwi genes in *An. albimanus* are predicted to encode two *Piwi* orthologs and no *Aub* ortholog. Four Piwi genes were identified for *An. minimus*, whose genome encodes two putative *Ago3* orthologs in addition to *Aub* and *Piwi* orthologs, and *An. dirus,* whose genome encodes two putative *Piwi* orthologs in addition to *Ago3* and *Aub* orthologs (Figure 2B). When amino acid sequences from all seven *Aedes aegypti* Piwi subfamily members were included in the analysis, *Aub*-like and *Piwi*-like orthologs segregated as predicted in earlier reports (Figure 1B; Akbari *et al.* 2013). A higher amino acid sequence diversity at the N-terminus of the *An. stephensi* piRNA components is consistent with findings in *D.*

melanogaster (Brennecke et al., 2007); therefore these sequences were used as the basis for designing corresponding probes for hybridization *in situ*.

Stage- and tissue-specific transcript abundance

Quantitative real-time PCR was used to detect and measure the accumulation of *AsAgo3*, *AsAub* and *AsPiwi* transcripts at embryonic, larval, pupal and adult developmental stages, and in ovaries and carcasses of adult females (Figure 3). All three piRNA pathway gene transcripts are most abundant at 48-72 hours post blood meal (hr PBM) in the ovaries and also are significantly more abundant in early embryos (0-2 hr) than at any other stage analyzed (P-values are listed in Appendix 1). This expression profile is consistent with microarray data collected for these genes in *An. gambiae*, although in those experiments, transcript abundance for all three genes increases significantly by 24 hr PBM (p-values=0.00005, 0.0021, 0.006 for *AgAgo3*, *AgAub* and *AgPiwi,* respectively: Marinotti *et al.* 2005). Transcription of zygotic genes does not occur earlier than 1-2 hours post egg laying in fertilized embryos of *D. melanogaster* (Pritchard & Schubiger, 1996; Zalokar, 1976). Assuming a similar regulation of the zygotic genome exists in *An. stephensi* (Biedler *et al.*, 2012), transcripts present in early embryos represent those deposited maternally during ovary development. These combined data support the hypothesis that *AsAgo3, AsAub,* and *AsPiwi* genes are expressed at the appropriate time and place to repress transposon expression and remobilization.

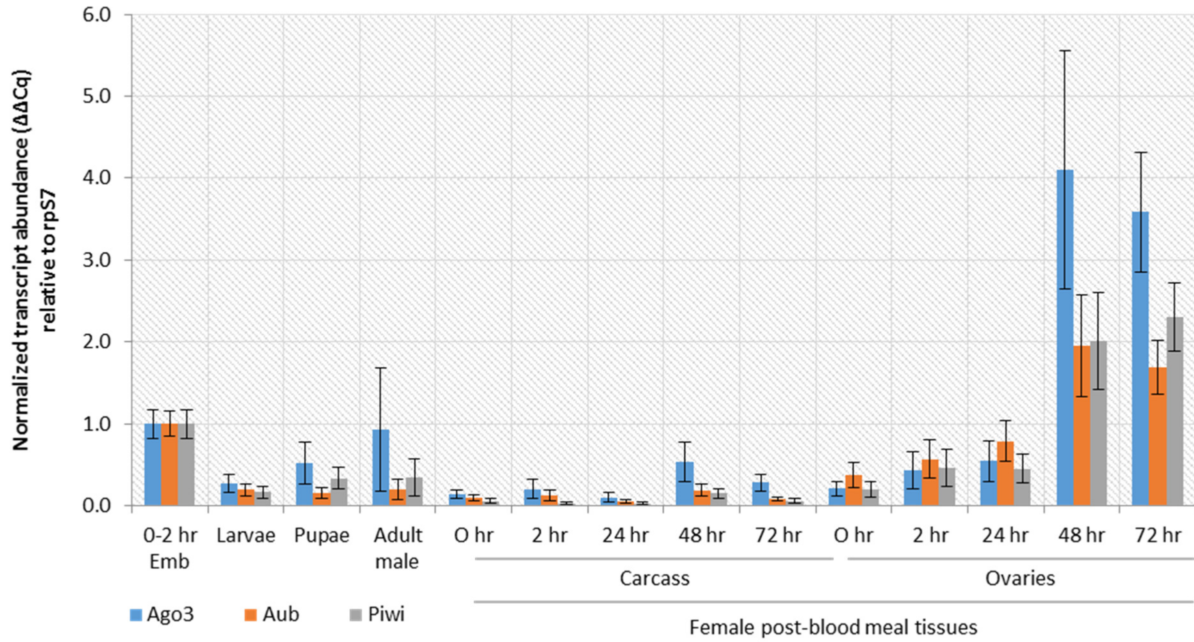


Figure 3. Abundance profiles of AsAgo3, Aub and Piwi transcripts during development. Each histogram represents the data (average ± SEM) of three biological replicates normalized to the embryo sample. Adult female 0 hr PBM samples were collected before blood-feeding. Embryos were collected between 0 and 2 hours following oviposition. P-values for all comparisons are listed in Appendix 1.

The localization of Piwi gene transcripts was determined at different times during ovary development. *AsAgo3, AsAub* and *AsPiwi* antisense RNA probes hybridized to transcripts in the cytoplasm of the nurse cells and oocytes of primary follicles, collectively the germ-line tissue, and in the previtellogenic secondary and tertiary follicles, which represent the earliest visible stages of oocyte development, that do not progress further until subsequent blood meals (Figure 4). Diffuse signals corresponding to the three piRNA pathway gene transcription products are seen in oocytes at 24 hr PBM. These signals are barely distinguishable visibly at 36 hr PBM and undetectable at 48 hr PBM. Since transcript abundance measured by quantitative gene amplification (qPCR) at this stage is 2-4 fold higher than in ovaries from unfed females, we speculate that the transcripts in the oocytes at 48 hr PBM are still present but either too diffuse to detect using hybridization *in situ* or that the endochorion at this stage is developed enough so that the hybridization and/or detection components of the assay could not penetrate the primary follicle. Furthermore, no transcripts were detected in either experimental or control (sense-probe) groups of embryos collected 0-2 hours following oviposition, although this stage also has abundant transcripts based on qPCR analyses. These transcripts likely are those deposited into the oocyte by nurse cells during development. It is also evident from these images that by 48 hrs PBM, the secondary follicles represent an important contributor to the transcript quantities measured in qPCR, indicating that new transcript present at this time point is not likely in the primary follicle. It is reasonable, based on the data presented here, to hypothesize that *AsAgo3, AsAub* and *AsPiwi* are expressed early in the primary, secondary and tertiary follicles following a blood meal and that their transcripts are accumulated and present in the oocytes throughout development. Strong, nonspecific background staining

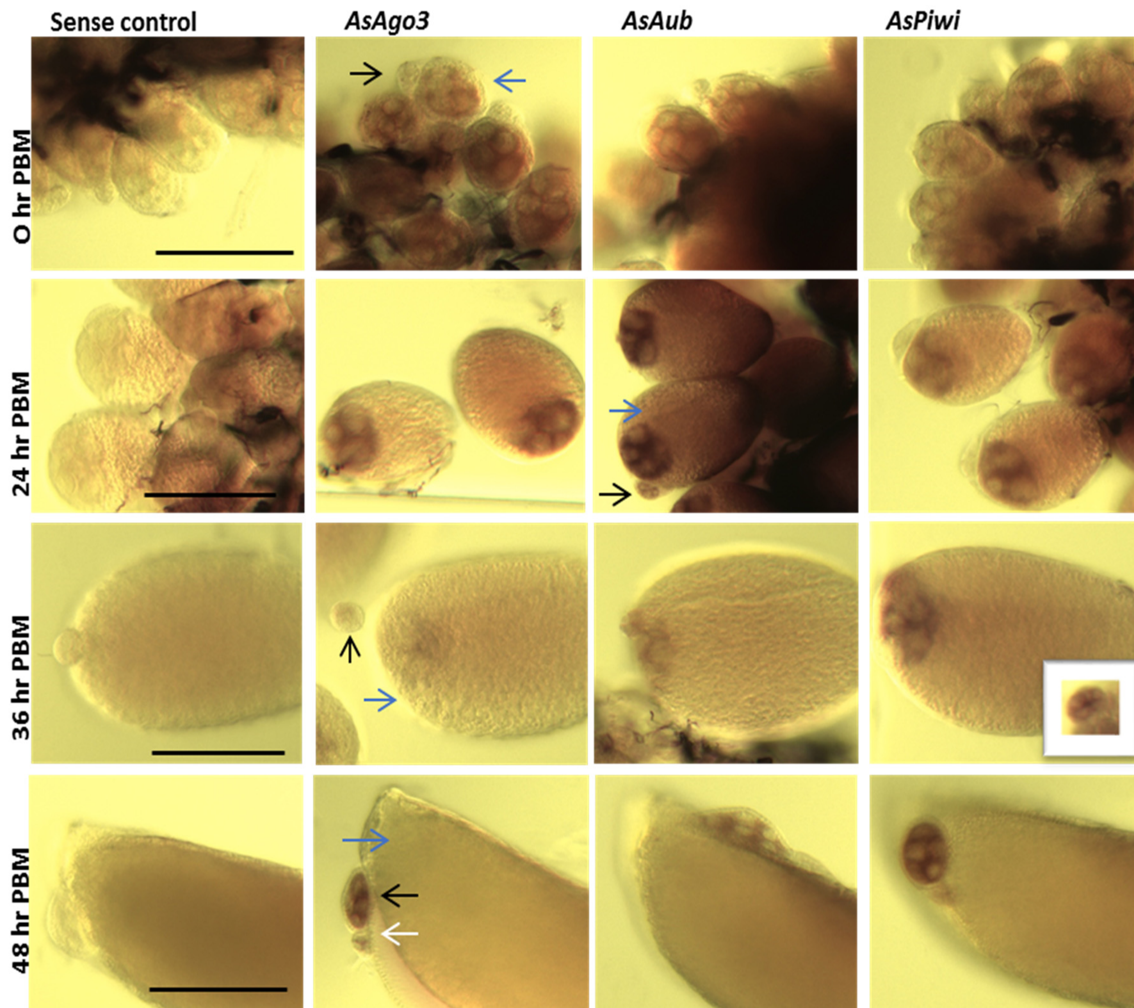


Figure 4. Spatial localization of *AsAgo3, Aub* and *Piwi* transcripts in ovaries. Whole-mount hybridization *in situ* of *An. stephensi* ovaries in sugar-fed (0 hr PBM) and at 24, 36 and 48 hr PBM. Blue, black and white arrows indicate the primary, secondary and tertiary follicles, respectively. Scale bar depicts 100 micrometers for each temporal group. Inset in 36 hr PBM *AsPiwi* samples, is a shifted focus to show signal in the secondary follicle.

was seen at 48 hr PBM superficially around the egg floats and at all stages in the trachea in both control and experimental groups.

These combined data support the conclusions that piRNA pathway gene transcripts in *An. stephensi* are abundant and enriched in the germline tissue during follicle development and are present in newly-laid embryos. These expression properties are consistent with a role in germ-line protection from transposon mobilization. Future experiments will focus on addressing whether transposon repression is indeed a role of the piRNA pathway in this important vector mosquito species.

Methods

An *Anopheles stephensi* strain maintained in our laboratory since 2004 was founded with mosquitoes provided by Dr. Marcelo Jacobs-Lorena (Johns Hopkins University). This line was used for all experiments reported here. Mosquitoes were maintained at 27°C and 77% humidity with 12:12 daily light-dark cycles and 30 minute dusk and dawn transitions. Larvae were fed a diet of powdered fish food (Tetramin) mixed with yeast. Adults were provided 10% sucrose *ad libitum*. Anesthetized mice were used for blood feeding adult females.

Samples for qPCR analyses were prepared from whole mosquitoes, dissected ovaries or carcasses (all tissues excluding the ovaries). RNA was extracted from 50 individuals for each sample (except embryos, where ~300 were used) by homogenization in Trizol reagent (Invitrogen) followed by chloroform extraction and RNA purification

using the Zymo Clean and Concentrator 25 (Zymo Research). Samples were treated with DNase RQ1 (Promega) and tested for genomic DNA contamination. Complementary DNA (cDNA) was synthesized using the iScript kit (Biorad) and used directly for qPCR reactions with Kapa Sybrfast supermix (Kapa Biosystems). All primers were optimized for annealing temperatures and cDNA concentrations; at least three technical and biological replicates each were used for each data point. Primers and corresponding amplification efficiencies are listed in Appendix 2. Biorad software (Version 3.0) was used for statistical analysis with the default two-sided t-test with a p-value threshold of 0.025 set for significance (Appendix 1-1).

Rapid Amplification of cDNA Ends (RACE) was performed with the SMARTer RACE kit (Clontech). cDNA was synthesized using the Clontech reagents and RNA collected from ovaries dissected 48 hr PBM. Gene amplification reactions were performed using Phusion High Fidelity Master Mix from New England Biosystems. Nested RACE reactions were performed with touchdown-PCR cycles on separate preparations of 5'- and 3'-end cDNA templates as described in the Clontech protocol. RACE and nested-RACE products were run on agarose gels and selected amplicons were cloned into the pCR-Blunt II TOPO plasmid. Plasmids were transformed into and amplified in chemically-competent TOP10 *E. coli*, and sequenced using M13 forward and reverse primers. Consensus mature transcript sequences have been deposited in Genbank with accession codes KJ808821 (PiwiA), KJ808822 (PiwiB), KJ808823 (Ago3) and KJ808824 (Aubergine).

Predicted amino acid sequences of *Ago3, Aubergine* and *Piwi* were downloaded from Vectorbase.com for all the *Anopheles* species available and for *Aedes aegypti*. Orthologous sequences of *D. melanogaster* were obtained from NCBI (http://www.ncbi.nlm.nih.gov). The resulting alignment was fed to MrBayes to simultaneously test 10 models of amino acids evolution (Ronquist et al., 2012). Phylogeny analyses were performed with RAxML through the Cipres Gateway Portal imposing the WAG mutation model and 1000 bootstrap resamplings of the original datasets. CONSENSE (PHYLIP version 3.5c, Felsenstein, 1993) was used to generate an unrooted consensus tree that was visualized by Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Hybridization *in situ* of ovaries was performed according to the protocol described by Juhn and James (2012). Briefly, ovaries were dissected from 5-10 mosquitoes and fixed in a 4% formaldehyde solution for 1 hr. Samples were treated with proteinase K, post-fixed and hybridized using digoxigenin (DIG)-labeled antisense and sense-RNA probes generated with the DIG RNA-labeling kit (Roche). The 5' RACE product clones were used as a template for amplification with M13 forward and reverse primers and each PCR product was used as a substrate for the RNA-labeling reactions. Following overnight hybridization with the labeled probe, samples were treated with RNase A to remove unbound probe and incubated with anti-DIG-alkaline phosphatase (AP)-conjugated antibody (Roche) overnight at 4°C. Colorimetric detection of probe localization was performed by incubation with NBT/BCIP (Roche) as a substrate for 5-7 hours in the dark. Samples were incubated overnight in glycerol, mounted and visualized using bright-field microscopy.

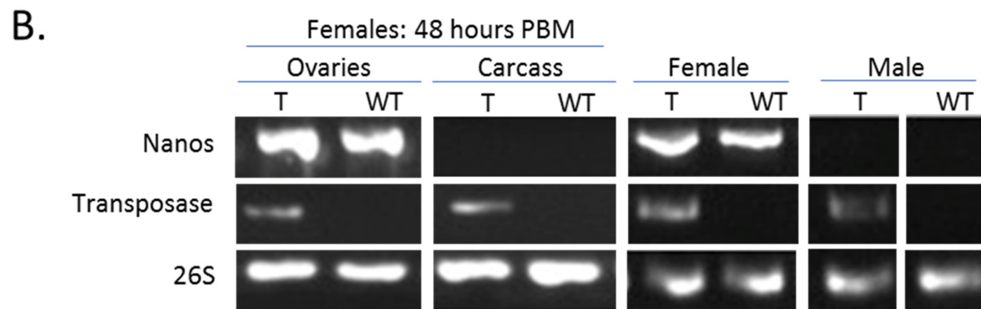
Chapter 3

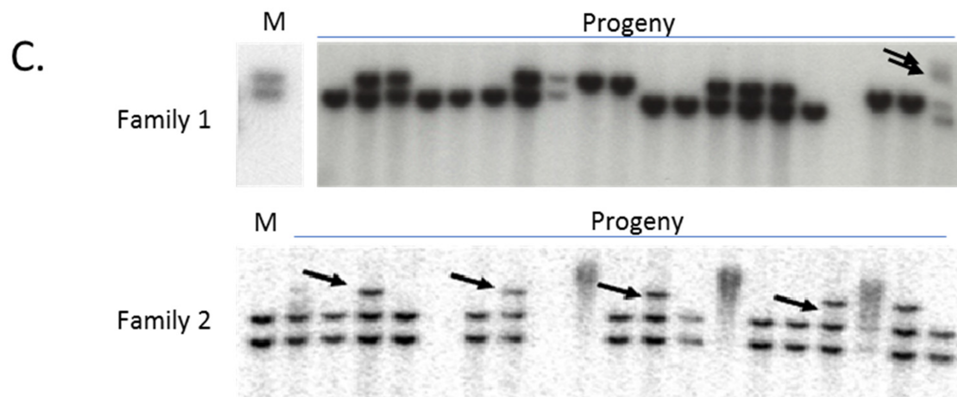
Characterization of Mobility of a Synthetic Autonomous Transposon

James *et al.* proposed that anti-pathogen effector genes could be encoded synthetically to be driven by endogenous promotors so that transgenic mosquitoes could express these anti-pathogen genes in tissues and at times relevant to pathogen clearance (James, Blackmer, & Racioppi, 1989). This idea spawned decades of work by many that lead to mosquito transformation, characterization of genetic control elements and ultimately a mosquito strain that blocks the complete development of the malaria parasites to prevent transmission to a human host (Isaacs et al., 2011, 2012). The idea was based on the observation that transposable elements could mobilize from one DNA location to another and that there were a defined set of requirements for mobilization that could be encoded synthetically with non-transposon genes (Spradling & Rubin, 1982). This remarkable characteristic also is the basis for the proposition that such a synthetic transposon construct could be encoded to self-mobilize and act as a gene-drive to move the anti-pathogen gene cassette into a population (Kidwell & Ribeiro, 1992; Ribeiro & Kidwell, 1994).

As a first step towards generating such a construct, the *piggyBac* transposon was used as a basis for a construct that encoded the green-fluorescent protein (GFP) marker and the *piggyBac* transposase under the control of the *An. stephensi nanos* promoter and 3'UTR (Figure 5A). The *nanos* control elements drive expression of the transposase in the ovaries (Figure 5B) (Calvo et al., 2005). This construct was used to transform *An. stephensi* and several integration events led to a line with four separate copies of the construct. RT-PCR

A.
L
3XP3-GFP-SV40
0.9nanos-Transposase-nanos 3'UTR
R


B.
Females: 48 hours PBM
Ovaries
Carcass
Female
Male
T
WT
Nanos
Transposase
26S


C.
M
Progeny
Family 1
Family 2

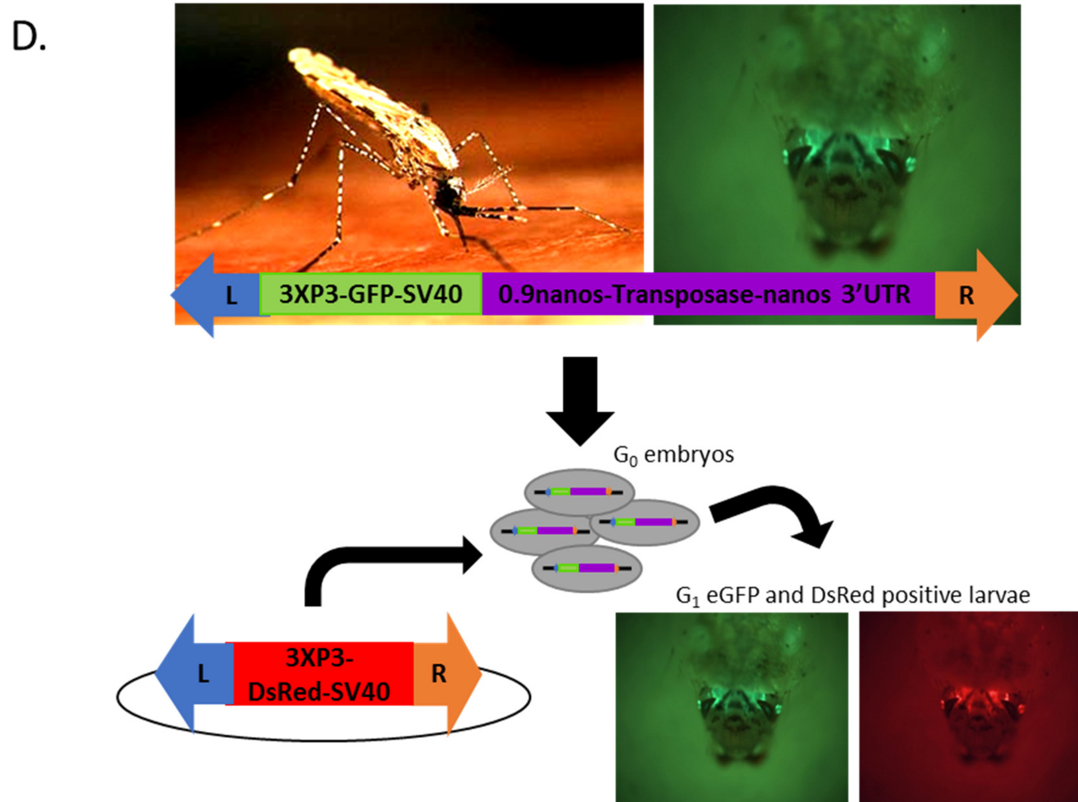

D.
L
3XP3-GFP-SV40
0.9nanos-Transposase-nanos 3'UTR
R
G0 embryos
3XP3-
DsRed-SV40
G1 eGFP and DsRed positive larvae

Figure 5: Endogenous transposase-mediated remobilization. A) Schematic representation of the piggyBac construct used to engineer a transgenic *An. stephensi* line expressing transposase driven by *nanos* control elements B) RT-PCR analysis of the presence of *nanos* gene transcript, *piggyBac transposase transcript* and 26S as a positive control in female ovaries and carcass and males. C) Southern blot analysis of 2 mothers and their offspring using a ^{32}P probe for eGFP. Diagnostic bands present in offspring but not mothers are indicated with arrows and represent remobilization events. D) Schematic of an assay for integration of a non-autonomous element. A construct encoding DsRed between *piggybac* arms was injected into embryos laid by eGFP-positive embryos without an exogenous transposase source, resulting in eGFP and DsRed positive larvae. Adapted from (Jimenez (2009)

analysis revealed that unlike the endogenous *nanos* gene, the transposase was expressed in all tissue assayed including female somatic and male tissues. This transgenic line was assayed for transposase activity by introducing an additional construct marked by *DsRed* expression in the eyes (Figure 5D). The *DsRed* marker gene was flanked by the *piggyBac* left and right arms, but no transposase was encoded on the construct and no exogenous source of transposase (usually a helper plasmid) was introduced during the microinjection. Integration of this second construct was observed showing that the transposase expressed from the transgenic mosquito was functional and active and so we expected that the construct would be able to self-remobilize. A series of Southern blot analyses were performed on individual outcrossed transgenic mother and offspring sets, and genomic DNA prepared from these were used to assess whether any insertions of the construct could be identified in progeny that were not present in the mother. Such insertions would be apparent by additional diagnostic DNA fragments or fragments differing in size and would indicate a potential remobilization event. Two remobilization events were observed in 386 progeny assayed from a total of 21 mothers resulting in a 0.5% remobilization frequency (Figure 5C).

In light of these observations, a new construct was generated with a number of improved characteristics. First, the final construct encoded two marker genes, *enhanced cyan fluorescent protein (eCFP)* and *DsRed* each flanked by a set of *piggyBac* arms, such that the marker genes were tightly linked and could be remobilized separately (Figure 6A). This enabled us to identify excision and remobilization events by visual inspection of the eyes instead of time- and resource-intensive Southern analysis. Second, the region upstream of

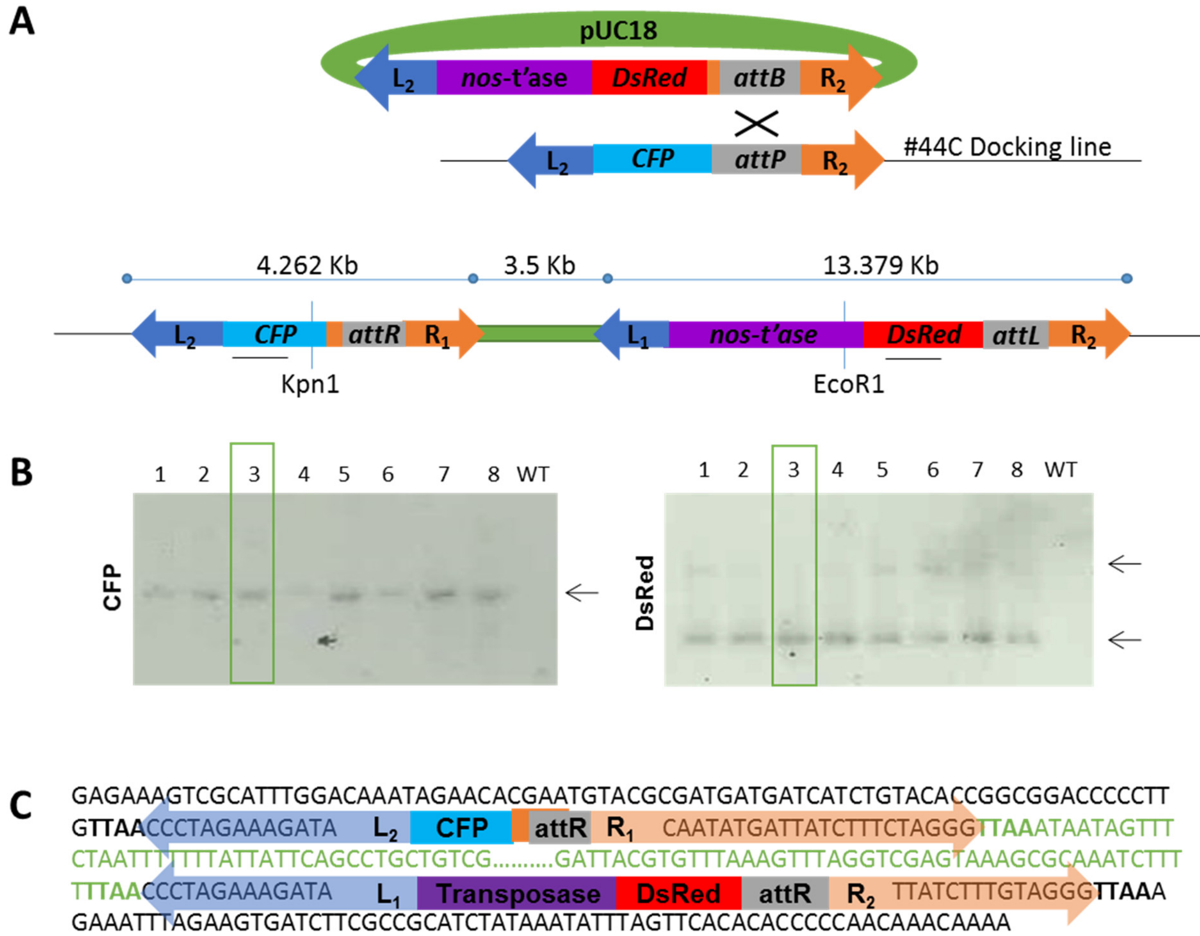


Figure 6: Generation and characterization of 3.8nanos-attP-44C-VM, a transgenic *An. stephensi* dual-reporter for transposon mobilization. A) Schematic representation of the transgenic design. *DsRed*, the *piggyBac transposase* open reading from flanked by 3.8 kb of the 5' region of *An. stephensi nanos* and the *nanos* 3'untranslated region (Nos-t'ase) and a *φC31 attB* site were cloned between *piggyBac* arms and the plasmid was used to transform attP-44C line with *φC31* integrase to generate two tightly-linked markers. B) Southern-blots probing for eCFP and DsRed in individual dual-reporter males to identify a founder with only the tightly-linked construct at 44C. Males were numbered arbitrarily and number 3 was selected to found the 3.8nanos-attP-44C line. Arrows indication bands diagnostic of a gene copy C) Sequence of DNA flanking *piggyBac* L_1, L_2, R_1 and R_2 arms at the 44C site, identified by inverse PCR and splinkerette PCR.

the *nanos* gene used to drive expression of the transposase was larger (3.8 kb compared to 0.9kb previously), which we hypothesized could increase the expression. We reasoned that as the control elements in the promoter are not characterized it is possible that by including more of the region 5' to the open reading frame, we were more likely to include all of the expression control elements, although ~1.5 kb of the 5'region upstream of *Ae. aegypti nanos* has been shown previously to be sufficient to drive abundant tissue-specific expression (Adelman et al., 2007). Third, a scheme was devised to integrate a plasmid using *φC31*-medited site-specific recombination, so that the construct would be present at a known and characterized location in the genome. The transgenic line bearing the construct as illustrated in Figure 6A was generated by microinjection of an *An. stephensi* docking line-44C (Amenya et al., 2010) and maintained by intercrossing.

Results and Discussion:

Isolation of 44C *φC31* integrase-mediated insertion genotype

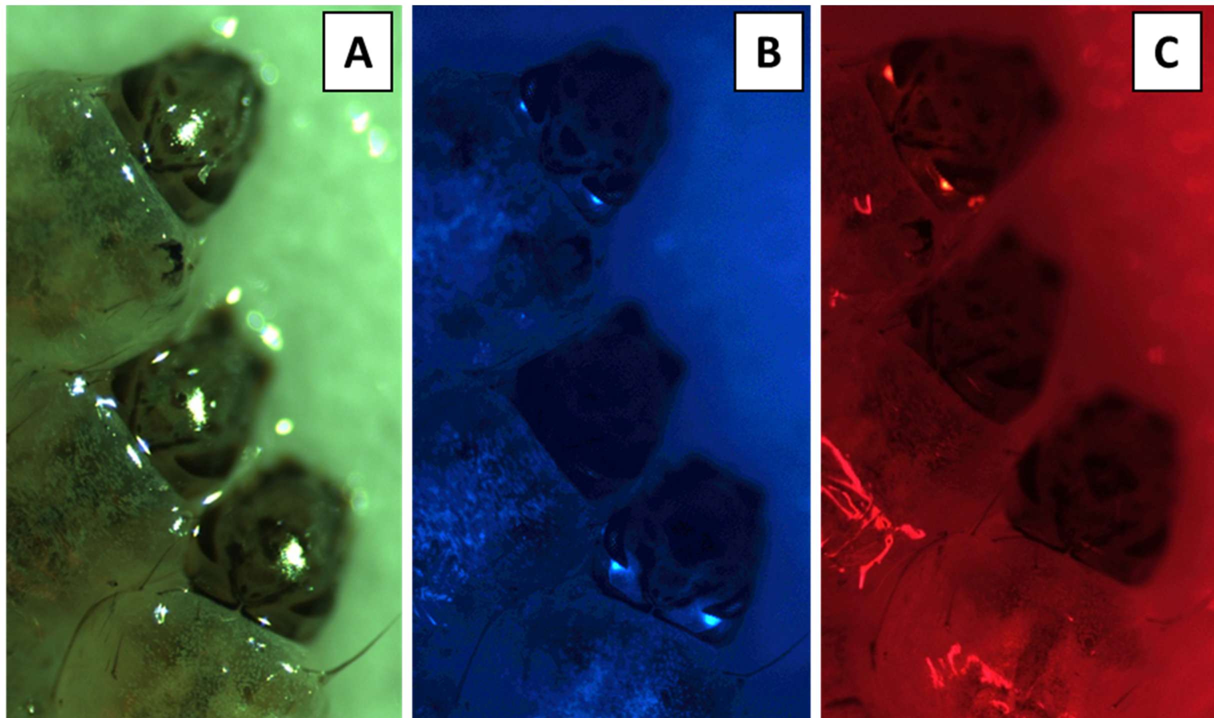
During the microinjection performed to establish the dual-transgene line, it is possible that transposase was expressed from the injected plasmid, which could have mediated remobilization of either the DsRed construct from the injected plasmid into the genome or the CFP construct from the 44C site into a new location in the genome. It was necessary to isolate the *φC31* integrase-mediated site-specific integration from genotypes resulting from G_0[2] *piggyBac* remobilization so that individuals identified as only eCFP-positive or only DsRed-positive in future generations represented remobilization from the 44C site mediated by endogenously-expressed transposase, not transposase expressed

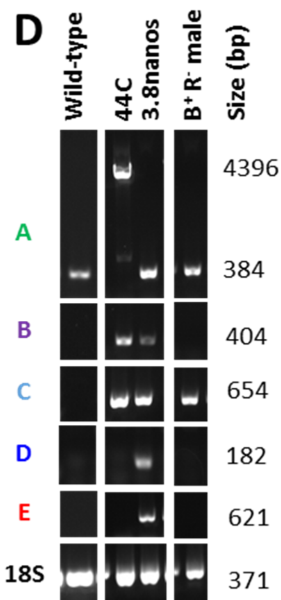
[2] In transgenesis terminology, G_0 organisms are those into which the transgenes are first introduced by injection or other means. G_1, G_2, etc. are subsequent generations.

during microinjection. In order to generate a line that would indicate such activity, eight males were outcrossed individually, and then assayed molecularly for their genotype. Male #3 was found to have only one copy of e*CFP* and *DsRed* by Southern analysis and inverse PCR showed that they were both present at the 44C site as originally intended (Figure 6 B,C). This male was outcrossed to found the line 3.8nanos-attP44C-VM.

Identification and characterization of a remobilization event

To identify remobilization events, males from the line were outcrossed at every generation and the progeny screened for exceptional phenotypes, which include mosquitoes showing only eCFP or DsRed in the eyes under fluorescence microscopy or eCFP- and DsRed-positive male individuals. The 44C site is located on the X-chromosome, so by outcrossing males, we can identify any male progeny with fluorescence as exceptional, since the original insertion should only be passed to females. Additionally, we expect that most stock females will bear the original insertion on both X-chromosomes since the line is maintained by intercrossing. In progeny from outcrosses of these females, remobilization events will be masked, since both *eCFP* and *DsRed* genes will be passed to all progeny. After five generations of outcrossing, no exceptional phenotypes were seen. We reasoned that the mobilization frequency could be influenced by transposase dose, so we began maintaining the line by intercrossing. Larvae were screened at every generation for several generations without identification of exceptional phenotypes.





E

Insertion onto Chromosome 2L

CAATTTGCCCCGTGCTCCGTTGCAGAAGGTGTGCAGCAGGT
GTGCAACTTCTCTCGCTACGGTACCAAGAAACCCTTCGCCA
GCCCATTTCCCAACTACCTTGAGTTCCGAGAACGGGCTCTG
TGCGGGAACGACATTATTTCATCGCCACTGGTGTTGGTAGG
GAACGGAAAAAAAGTTCTAGCGCAAACAGTGTGCTGTGGT
TTTT**TTAAC**CCGCG L_2 CFP attR R_1 AGGG**TTAAC**
CAGGTCAATATTCTATTTTTGTAGCATAAAACCTTTGAATTTT
GTTGCATATATACATTTTTTGCGAAATATTTGACGGTTTTTCA
GCGATGCTATTGTTTTTTTTTTTGAGTAACAAAGCGAATGTTA
TTAAACTTTTTGATAACTTTCTGCAATCAGTTTAACTAATTGT
TTTGATAGGAATATAATAGTATTGTTTTAGCGTGATTCAGTAA
AAAG

Figure 7: Screening for exceptional phenotypes by fluorescence microscopy. Mosquitoes of the 3.8nanos-attP-44C-VM line were screened for eCFP and DsRed in the eyes. From top to bottom in each picture: 3.8nanos-attP-44C-VM larva with fluorescent markers, a larva from the wild type colony, a 3.8nanos-attP-44C-VM larva with an exceptional phenotype (eCFP, but not DsRed). A) Bright field microscopy B) Fluorescence microscopy, eCFP. C) Fluorescence microscopy, DsRed.

After 28 generations, 11 males from the transgenic line were outcrossed and eCFP-only individuals were identified (Figure 7). In order to identify how many remobilization events had occurred over the past generations, a total of 30 males were collected from the colony at generation 37 and outcrossed to wild-type females. Offspring were separated by phenotype and 10 males of each phenotype were outcrossed to generate individual families to increase the amount of genomic DNA available for analysis. Individuals were analyzed first by PCR to verify the observed phenotype and to begin to characterize remobilization events. The PCR-primer scheme presented in Figure 8 allowed us to characterize individuals as excision or remobilization events. The presence of only the e*CFP* or *DsRed*-marked construct at the 44C locus indicates that an excision from the site had occurred; however since a remobilization event involves both an excision and integration into a new site, these events would not be included in the estimate of remobilization rate. Individuals that were classified as having evidence of remobilization were grouped by phenotype and aliquots from the genomic DNA of five individuals were pooled for inverse PCR. Only one remobilization event was captured; a remobilization of the eCFP-marked portion of the construct was identified by inverse PCR to be on chromosome 2L, scaffold_00100 (Indian Strain, VectorBase.org) (Figure 8C) at the TTAA sequence at position 272403-272406. TTAA sequences flanking the construct support the conclusion that the construct was precisely excised and re-integrated by *piggyBac transposase* (Fraser, Ciszczon, Elick, & Bauser, 1996). The identification of this remobilization event is important support of a proof-of-principle that a synthetic transposon construct can be designed to self-mobilize. It is not possible from these data to estimate a remobilization frequency, but one event detected over 39 generations is far below the remobilization rates predicted by early

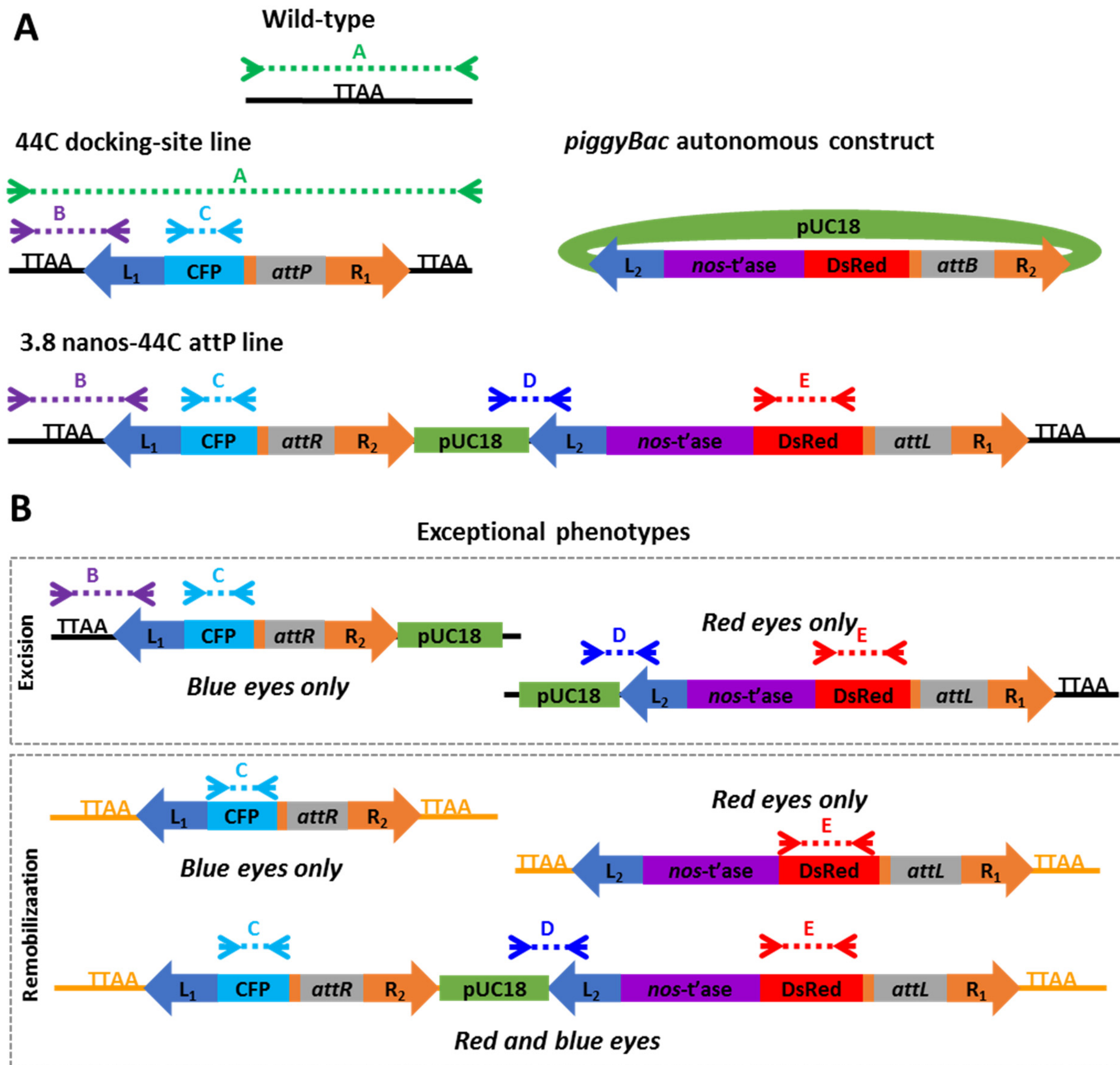


Figure 8. Schematic representations of gene structures and expected amplification products resulting from autonomous mobility of transgenes. A) The docking site line comprises a transgene consisting of the left (L_1) and right (R_1) inverted terminal repeats of the *piggyBac* transposable element flanking the coding sequence of the cyan fluorescent protein (CFP) driven by a 3XP3 promoter and sequences for site-specific recombination (*attP*) from the phage *φC31* (Isaacs *et al*., 2012) integrated into the genome (horizontal black line). *piggyBac* transposase-mediated insertion causes a target-site duplication (TTAA) flanking the transgene. *φC31* recombinase-mediated integration of a *piggyBac* autonomous construct comprising a cloning plasmid (pUC18) carrying an additional set of *piggyBac* left (L_2) and right (R_2) inverted terminal repeats, the *piggyBac* transposase coding sequence driven by the *nanos* promoter (*nos*-t'ase), the DsRed fluorescent protein coding sequence (DsRed) driven by a 3XP3 promoter and the *φC31 attB (attB*) site yields the 3.8 nanos-44C attP line. DNA derived from wild-type and 44C docking-site line mosquitoes

generates diagnostic amplification fragments of 384 and 4396 bp, respectively, when using primer set A. Similarly, primer sets B and C yield fragments of 404 and 654 bp, respectively, when DNA samples from 44C docking-site and 3.8 nanos-44C attP mosquitoes are used as templates. Primer sets D and E are specific to the 3.8 nanos-44C attP line and produce fragment of 182 and 621 bp, respectively. **B**) The generation of exceptional phenotypes (Blue or Red eyes only) can result from excision or remobilization of portions of the 3.8 nanos-44C attP transgene complex. Primer sets B, C, D and E are diagnostic for excision events in the combinations illustrated. Remobilization puts portions or all of the transgene complex at a new site in the genome (horizontal orange line). Inverse PCR techniques are used to identify the new genomic location.

models to be necessary for a useful gene-drive mechanism (M. Kidwell & Ribeiro, 1992; Rasgon & Gould, 2005; Ribeiro & Kidwell, 1994). In order for this technology to be useful, it is likely that several adjustments will need to be made to the construct. A more robust promoter that expresses *piggyBac* transposase at a higher level in the ovaries may increase the transposition frequency. It is also possible that the transposase is being post-transcriptionally regulated by the piRNA pathway. piRNAs are derived from regions of the genome that have remnants of transposons to which the organism was exposed previously in its life history. It may be that an ancestor of our *An. stephensi* line encountered an ancestor of the *piggyBac* transposon. A blast of the currently available annotated *An. stephensi* genomes produces regions of homology between 19 and 40nt in length with 86-100% homology to *piggyBac transposase* (VectorBase.org), but data are not currently available as to whether these regions are piRNA producers. It could be that ancient exposures to ancestral *piggyBac* transposons present on the genome provide enough sequence to establish repression of contemporary transposon. If indeed piRNA regulation was influencing transposase availability, it may be possible to redesign the construct to avoid this regulation by altering the nucleotide sequence. Since the regulation is mediated by nucleotide sequence specificity, if the transposase is encoded with different codons, the transposase transcript would essentially be invisible to the piRNA machinery.

In light of current efforts to develop gene-drive mechanism based on Cas9 activity (Gantz & Bier, 2015) it seems that other technologies may prove more robust for moving anti-pathogen transgenes into a population. However, at our current state, a gene-drive mechanism is still unavailable. It also is possible that inundative release of transgenic mosquitoes may be sufficient for the goal of fixation of an anti-pathogen genotype into wild

populations, but the benefit afforded by the elegance of a gene-drive technology in terms of reduced cost and effort of implementation over an inundative release strategy, makes developing the technology a worthwhile pursuit (Macias & James, 2015).

Methods:

The plasmids *p*Bac3XP3-GFP[0.9nanos-*p*BacORF] and *p*BacDsRed-attB[3.8nanos-*p*BacORF] were synthesized prior to this work and will be reported in Macias *et al*. 2015 (In preparation). Transgenic *An. stephensi* carrying *p*Bac3XP3-eGFP[0.9nanos-*p*BacORF] were created by injecting preblastoderm embryos with a mixture of *p*Bac3XP3-eGFP[0.9nanos-*p*BacORF] (300ηg/μL) and *piggy*Bac helper (200ηg/μL) plasmids as described (Catteruccia et al., 2000). Site-specific recombination was used to integrate the plasmid *p*Bac 3XP3-DsRed[3.8nanos-*p*BacORF] into an *attP* docking site by microinjection of the docking line attP-44C (Amenya et al., 2010; Isaacs et al., 2012) with a mixture of *p*Bac 3XP3-DsRed[3.8nanos-*p*BacORF] (300ηg/μL) plasmid and integrase mRNA (400ηg/μL) as described previously (Nimmo, Alphey, Meredith, & Eggleston, 2006). Pools of 3-5 G_0 males or up to 20 females were mated to wild-type mosquitoes and G_1 progeny screened.

cDNA was prepared from mosquito tissues as described in Chapter 2. Phusion polymerase used to amplify diagnostic products with adjusted cycling parameters. All PCR reactions were performed according to the manufacturer's protocol, with 60 second denaturing, annealing and extension steps. Primers are listed in Appendix 2.

For analysis by Southern blot, genomic DNA from individual mosquitoes was isolated using Wizard Genomic DNA Purification kit (Promega) and ~3.5 μg digested using 30U of *EcoR*I or *Kpn*I in a 20 μL reaction. Digested DNA was run on a 0.8% agarose in TBE gel at 70V for 5 hours or at 20V overnight (~16 hours). Gels were visualized after a 10 minute stain in a GelRed 3,000X TAE (Biotium), soaked in a denaturation solution (1.5M NaCl, 0.5 NaOH) twice for 15 minutes, and soaked in a neutralization solution (1.5M NaCl, 0.5M Tris-HCL (pH 7.2), 1 mM EDTA) twice for 15 minutes. Gels were rinsed with deionized water. DNA transfer to a nylon membrane was set up according to standard protocols (Sambrook, Fritsch, & Maniatis, 1989). Following transfer, nylon membrane blots were rinsed in 2X SSC and cross-linked at 1200μW/cm^2 in a UV Stratalinker (Stratagene). To generate GFP probes, the first 450bp of the eGFP open-reading frame (ORF) were cloned into a TOPO TA vector (Invitrogen), digested with *EcoR*I and the fragment extracted and purified from an agarose gel. A Southern blot also was used to identify males from the colony that had one copy of each of e*CFP* and *DsRed* marked constructs. Genomic DNA isolated from individual males was divided such that one-half was digested with *Kpn*I and probed with ^{32}P-labelled eCFP DNA probe, and the remainder digested with *EcoR*I and probed with the ^{32}P-labelled DsRed DNA probe. Labelled probed was generated by PCR amplification of a 600-700 bp fragment from plasmids bearing the marker genes. The PCR or digestion products were gel-electrophoresed, extracted and amplified with ^{32}P-labelled dATP and dCTP (Perkin-Elmer) using Megaprime DNA labeling system (Amersham).

For analysis by splinkerette PCR, genomic DNA was extracted from adult mosquitos using DNeasy Blood & Tissue Kit (Qiagen) or Wizard genomic DNA purification kit

(Promega) and digested with *Bst*I and splinkerette PCR was performed as described previously (Potter & Luo, 2010). PCR products were resolved in agarose gels; fragments amplified from the dual reported construct at the 44C site with the *piggyBac* 5' Rev primer were ~350 and ~400bp in length, respectively, . Any fragment at any other size was considered diagnostic for remobilization. All fragments were gel-extracted, purified and sequenced. Diagnostic PCR amplifications to identify the genotype of mosquitoes derived from the dual-reported line with exceptional phenotypes were performed with primer combinations as depicted in Figure 7 with primers listed in Appendix 2.

Inverse PCR was performed as described previously with primers listed in Table 3 (Handler, McCombs, Fraser, & Saul, 1998). Inverse PCR and Splinkerette PCR are analogous procedures that provide the same information, however we found that Splinkerette PCR was consistently effective for identifying the DNA flanking the *piggyBac* Left arm, whereas Inverse PCR was most robust for identifying the DNA flanking the *piggyBac* Right arm. The Inverse PCR protocol was performed initially to identify individual mosquitoes that had been observed to have only one fragment for each eCFP and DsRed probing of the genomic DNA by Southern blot. Following screening for exceptional phenotypes, genomic DNA was extracted from individuals, then inverse PCR was performed on pools of genomic DNA aliquots from individuals of the same phenotype. Digests were performed with either *Hea*III or *Msp*I for two hours and purified by ethanol precipitation. Ligations were performed using T4 ligase (NEB) with a total volume of 400 μL, overnight at 4°C. DNA from ligation reactions was purified using ethanol precipitation and used as a template for a PCR with primers listed in Appendix 2. PCR product was run

on an agarose gel and diagnostic fragments were extracted using QiaQuik Gel Extraction kit (Qiagen) and cloned and transformed using StrataClone Blunt PCR Cloning kit (Agilent Technologies). Resulting bacterial colonies where picked and grown for plasmid amplification and plasmid DNA was isolated using Zyppy Plasmid Miniprep kit (Zymo) and sent for sequencing by Laguna Scientific, a local fee-for-service company.

Chapter 4

The Impact of Stress on the piRNA Pathway and Transposon Mobility

During the course of analyzing the qPCR data collected from tissue and time points reported in Chapter 2, one embryo sample showed exceptionally high levels of transcript accumulation for *Ago3, Aub* and *Piwi* when compared to the product of the *ribosomal protein S7*, gene (*rps7)* (Figure 9). The data for this embryo sample were not included in the analysis for developmental assessment of Piwi gene expression presented in Chapter 2, since it was a clear outlier, however, it was noted that these embryos were collected from females that had been reared in our insectary during a temperature failure. We hypothesized that the increase in Piwi gene expression could be a temperature-induced response to stress.

The hypothesis that a link between stress and the piRNA pathway exists draws support from reports that link various forms of stress and transposon transcript abundance and mobility (Chen *et al.*, 2003; Feng, Leem, & Levin, 2013; McClintock, 1987; Pecinka & Scheid, 2012; Ratner, Zabanov, Kolesnikova, & Vasilyeva, 1992; Sehgal, Lee, & Espenshade, 2007; Strand & Mcdonald, 1985; Todeschini *et al.*, 2005). It could be that the piRNA pathway is active in response to increasing transposon activity as the activity of piRNA pathway proteins is certainly stimulated by the presence of transposon transcript (Brennecke et al., 2007), although it has not been reported that this activity leads to an increasing synthesis of the pathway components.

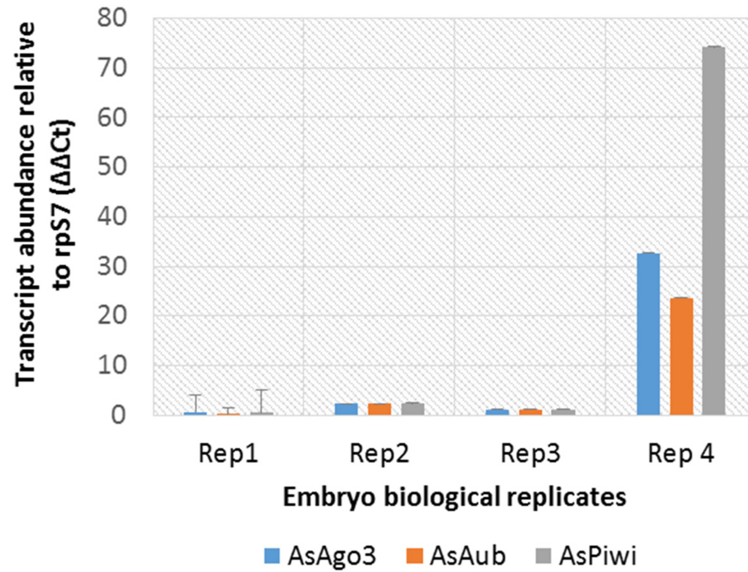


Figure 9: An exceptional replicate. Quantitative PCR analysis of four samples of ~300 embryos. Error bars are corrected SEM. Replicate 1-3 are the same analysis presented in Figure 3. Replicate 4 was omitted from the previous analysis, since it is a clear outlier.

Alternatively, the Piwi genes could be activated directly under stress. It seems intuitive that an organism that could restrain the increasing activity of genome-damaging transposons would be selected for under stress over those that could not mount such a response. However, it has been postulated that evolution would likely favor an overall phenotype that allows a certain level of transposon activity under stress as this could provide a selection medium by generating variation due to insertion-based mutations caused by transposon mobility (Pecinka & Scheid, 2012). These authors speculated that continuous stress could allow mutations to occur over some number of generations and the selection occurs in such a way as to yield an organism that no longer responds to the stress as stress. At this point, the restraints on transposons would be re-established. Could it be that a large quantity of *piwi*, *aub* and *ago3* mRNA deposited in the embryo, as seen in the one exceptional embryo sample, is a mechanism to re-establish this control in a future generation that is no longer interpreting a stress signal? The hypothesis that stress induces the expression of the piRNA pathway genes was tested by exposing mosquitoes to different forms of stress and measuring the response of *ago3*, *piwi* and *aub* and the heat-shock protein gene *hsp90* by qPCR analysis.

Results and Discussion

Hsp90 expression during development

The level of *hsp90* transcript in experimental and control mosquitoes was assayed to characterize a response in mosquitoes exposed to various forms of stress. The putative *An. stephensi* ortholog for *hsp90* was identified based on sequence homology to *An. gambiae hsp90* and qPCR primers were designed to the predicted transcript. Quantitative PCR analysis on developmental stages and post-blood meal tissues revealed interesting phenotypes in *hsp90* expression (Figure 10). An ~5.5-fold increase in *hsp90* transcript abundance is detected at 2 hr PBM in carcasses (adult female bodies without ovaries), but the transcript is induced even more highly in the ovaries, ~28-fold, at the same time point. Benoit *et al*. 2011 demonstrated that *Ae. aegypti* and *An. gambiae* undergo heat-stress in response to a blood-meal, which could explain this increase. The transcript abundance decreases at 24 hr PBM following this initial peak but interestingly increases again about 7-fold at 48 hr PBM only in ovary samples when follicles are fully developed. This is consistent with expression data in *D. melanogaster*, where the homolog to *hsp90*, *hsp83*, is expressed in ovarian nurse cells and deposited into the developing oocyte (Zimmerman, Petri, & Meselson, 1983).

The impact of *in-utero* heat stress on Piwi gene product abundance in embryos

In an attempt to reproduce the effects of stress on Piwi gene product abundance, adult females were exposed to one-hour of heat stress at 40°C at 4 days after a blood-meal when embryos are ready to be laid. Oviposition was induced by confining 10 females in a small

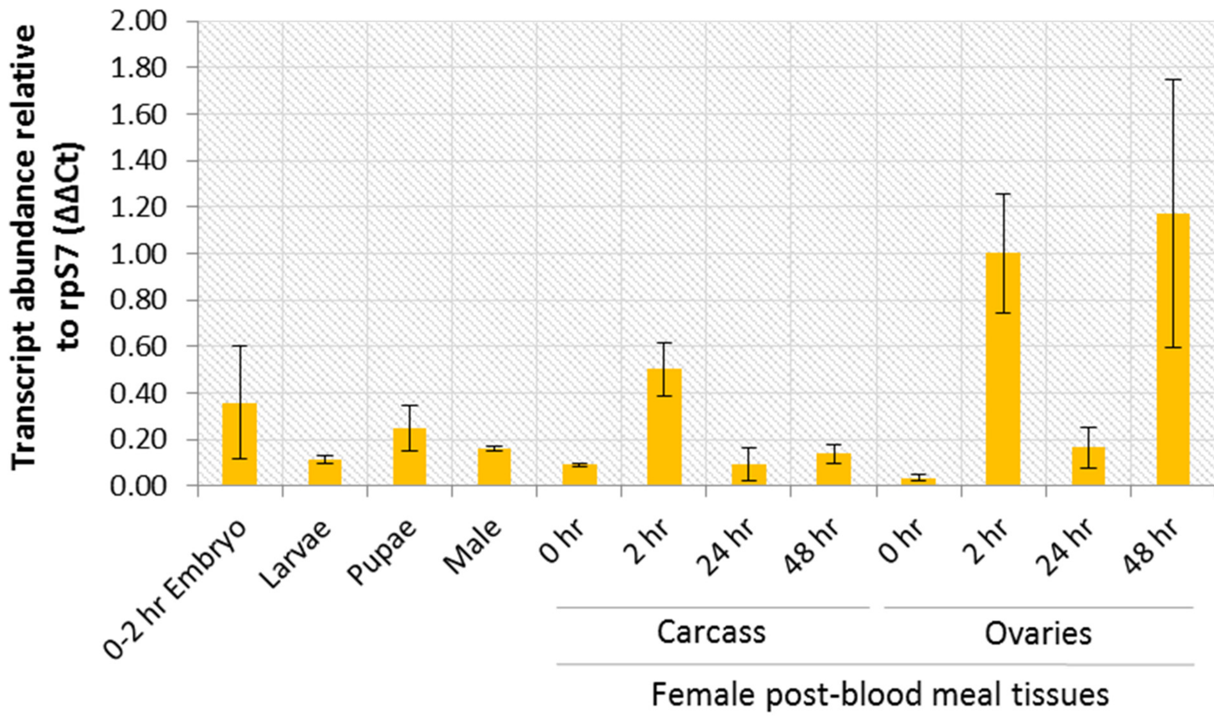
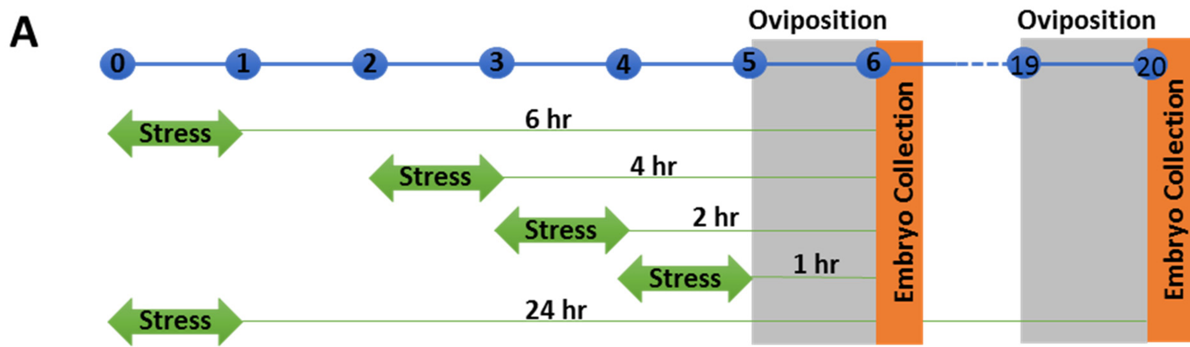


Figure 10: *hsp90* expression over development. Quantitative PCR analysis of *hsp90* transcript at different stages of development and in females after a blood meal. Values are ΔΔCt and relative to ribosomal protein gene rpS7.

tube with oviposition medium (wet cotton and Whatman paper) at different time points following the heat stress (Figure 11A). To collect the one-hour time point, small Petri dishes with wet cotton and filter paper were placed in the cage during the heat treatment. Interestingly, almost all females provided an oviposition substrate deposited embryos immediately. At least 300 embryos were collected from each treatment and control group. Three replicates of this experiment were performed on mosquitoes collected from our colony at different generations. Quantitative PCR revealed that *hsp90* expression was induced differently in every replicate heat-treatment group (Figure 11B). *hsp90* transcript level in the first and second replicate are lower than the third replicate in control mosquitoes and reach peak product abundance two hours after heat treatment compared to 1 hour for the third replicate, but the gene was induced to a similar level of abundance for all three replicates. Similar variation is seen in Piwi gene transcript abundance among biological replicates and for each replicate the increase in Piwi gene transcript abundance is similar to that of *hsp90*. A trend of increasing abundance with heat-shock is present although statistical analysis does not support a significant increase in transcript abundance for any of the Piwi genes. Importantly, none of the replicates reproduced the 20-80 fold increase seen in the outlier shown in Figure 9. The remaining collection of experiments represents an attempt to identify an effect of stress on the Piwi genes and transposon activity.



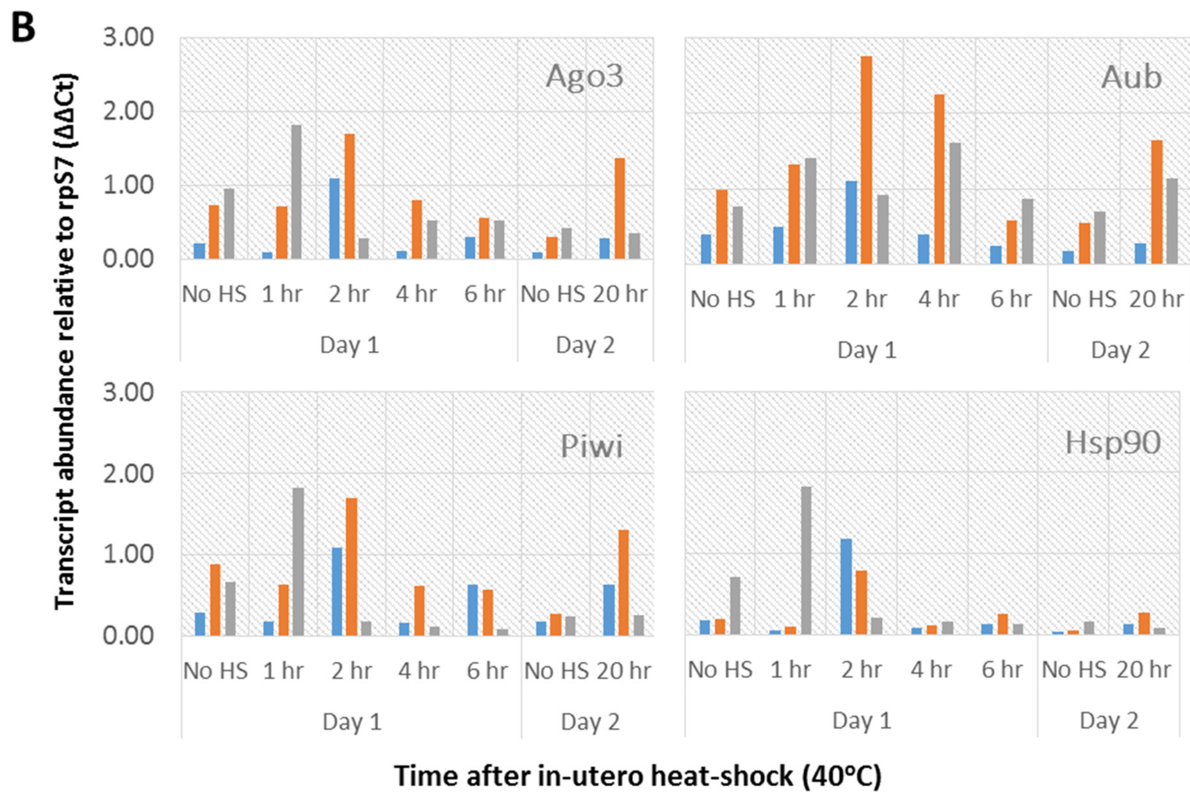


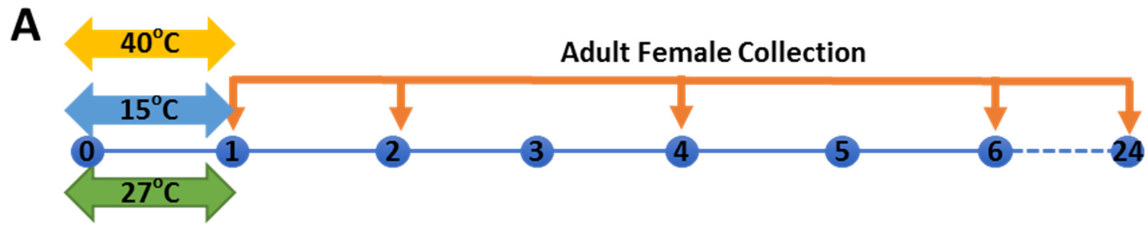
Figure 11: Induction of *hsp90* and Piwi gene expression in embryos following in-utero heat shock. A) Design scheme for *in-utero* heat shock. Females were exposed to 40°C heat-shock at 4 days post-blood-meal. Females were allowed to oviposit for one hour and 0-1 hour post-oviposition embryos were collected. B) Quantitative PCR data for three individual repetitions of the experiment outlined in A including control embryo samples collected each day (No HS). Values are ΔΔCt and relative to ribosomal protein gene *rpS7*.

Heat and cold stress in adults

Embryos were analyzed for Piwi gene transcript abundance in an effort to reproduce the increase seen in one early embryo sample (Figure 9). However, working with adult non-blood fed mosquitoes has many benefits over collecting embryos, mostly in terms of handling and timing of collection. To test whether increased Piwi gene product abundance is a phenomenon recapitulated in the whole adult mosquitoes, adult females were subjected to a 40°C heat shock or a 15°C cold shock for one hour with collections at several time points following stress (Figure 12). A significant change in *hsp90* transcript abundance was seen at all time points following heat stress , which occurred within the first hour and then decreased. *hsp90* transcripts also were significantly differentially accumulated at 4 hrs after cold-treatment. *Aub* transcript appeared to increase after heat-shock, but the change was not significant. In cold-shocked mosquitoes, all three Piwi genes appear to have increased product abundance; however these changes also are not significant when compared to transcript abundance in corresponding room temperature samples (27^{0}C). In contrast, all three Piwi transcripts showed modest, but significantly reduced abundance at a number of time points following stress.

Maternal follicle cells and nurse cells express transcripts that are deposited into the developing oocyte during development. Following oviposition, the embryo is thought to be mostly transcriptionally inactive (Biedler *et al.*, 2012; Pritchard & Schubiger, 1996), so it stood to reason that an increased abundance of *piwi, ago3* and *aub* in the embryo was the result of overexpression of the transcript in the ovaries. To test this, females were heat-shocked both at 24 hr PBM meal, when the eggs are still in development and at 72 hr PBM,

A



B

Figure 12: Piwi gene and *hsp90* expression whole adult females. A) Heat treatment and collection scheme. Five female mosquitoes were collected at 1, 2, 4, 6 and 24 hours following treatment at 40°C or 15°C. B) qPCR analysis of heat-treated, cold-treated and control mosquitoes. Values are ΔΔCt, relative to *rpS7* transcript abundance and normalized to 24-hour carcass value. Error bars are corrected SEM. One and two asterisks indicates p-values less than 0.5 and 0.001 respectively from a two-sided t-test comparing means of 3 stress-treated biological replicates at a time point with the mean of corresponding control biological replicates at the same time point.

when the eggs are developed fully and ready to be laid (Figure 13A). This analysis revealed that the increase in *hsp90* transcripts that was seen in whole adult females occurs almost entirely in the carcass, which represents all tissue except the ovaries (Figure 13B). The change in Piwi gene product abundance between carcass and ovary samples over development was consistent with that reported in Chapter 2. Contrary to what was expected, expression of the Piwi genes appears to be decreased with heat treatment.

Stress and transposon activity

We were able to gather some preliminary evidence that stress-induced mobilization could be occurring in *An. stephensi* by analyzing RNA seq data collected before and after a blood-meal. RNA sequences from *An. stephensi* female midguts before and 4 hr PBM were used to query the TEFam database, a collection of transposon sequences from *Aedes aegypti*, *Anopheles gambiae* and *Culex pipiens* (http://tefam.biochem.vt.edu/tefam/) (J. Biedler & Tu, 2003). A heat-shock response is induced in *Ae. aegypti* and *An. gambiae* following a blood-meal and it may be that transposon sequences expressed following a blood-meal may represent stressed-induced transcription (Benoit et al., 2011). Fifteen RNA sequences were identified with high identity (79-86%) to transposons identified in *An. gambiae, Ae. aegypti,* and *Culex pipiens* (Table 2); none were identified in analysis of sequences collected from non-blood fed females. Many types of transposons are represented, including Type II transposons, (the type used for mosquito transgenesis), non-LTR retrotransposons and LTR transposons. Notably, two sequences with high homology to Ty1-copia elements in other mosquitoes were identified. Stress studies in *D. melanogaster* revealed that a *copia*

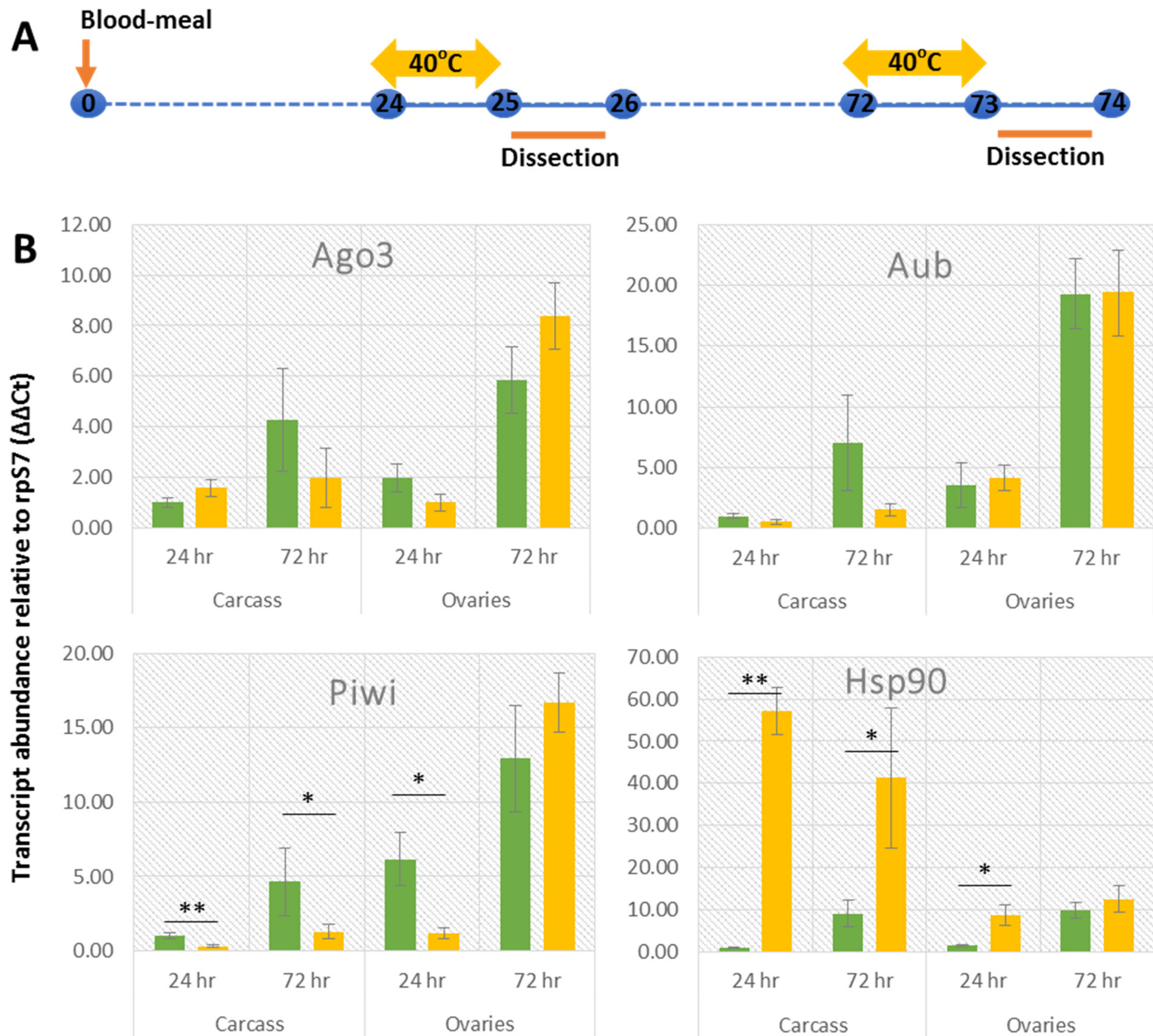


Figure 13: Piwi gene and *hsp90* expression in ovaries and carcass following heat-shock in blood-fed females. A) Heat treatment and dissection scheme. Approximately 25 female mosquitoes were dissected for ovaries and the remaining tissue (carcass) between one and two hours following treatment at 40°C. Treatment and dissection were performed at 24 and 72 hr PBM. B) qPCR analysis of heat-treated and control tissues. Values are ΔΔCt, relative to *rpS7* transcript abundance and normalized to the 24 hr PBM carcass value. Error bars are corrected SEM. One and two asterisks indicate p-values less than 0.5 and 0.001, respectively, from a two-sided t-test.

Table 2: Transposons identified with homology to transcripts present in 4 hr post blood meal *An. stephensi* females

TEfam code	Transposon name	Type	Organism	Quary length	Score	e-value	Identity
TF000631	Ty1_copia_Ele19	LTR Retrotransposon	Ae. aegypti	1659	1326	0	83%
TF000085	RTE_ele1, Jammin-1	Non-LTR Retrotransposon	An. gambiae	2534	75	5.00E-13	98%
TF000085	RTE_ele1, Jammin-1	Non-LTR Retrotransposon	An. gambiae	3797	881	0	82%
TF000085	RTE_ele1, Jammin-1	Non-LTR Retrotransposon	An. gambiae	2243	881	0	82%
TF000701	Ty1_copia_Ele60	LTR Retrotransposon	Ae. aegypti	4535	968	0	78%
TF001352	RTE_Ele2, Jammin-1	RTE Non-LTR Retrotransposon	An. gambiae	310	327	4.00E-90	86%
TF001353	RTE_Ele2, Jammin-1	RTE Non-LTR Retrotransposon	An. gambiae	496	169	4.00E-42	86%
TF001354	RTE_Ele2, Jammin-1	RTE Non-LTR Retrotransposon	An. gambiae	1862	169	2.00E-42	86%
TF000918	ITmD37D_ele6	DNA transposon TC1-Mariner Superfamily	Ae. aegypti	2652	169	2.00E-41	84%
TF000085	RTE_ele1, Jammin-1	RTE Non-LTR Retrotransposon	An. gambiae	666	141	1.00E-33	92%
TF000085	RTE_ele1, Jammin-1	RTE Non-LTR Retrotransposon	An. gambiae	398	193	2.00E-49	79%
TF0001798	RTE_ele2	RTE Non-LTR Retrotransposon	C. pipiens	398	52.8	3.00E-07	100%
TF0001797	RTE_Ele1	RTE Non-LTR Retrotransposon	C. pipiens	398	49.1	4.00E-06	97%
TF000084	Loner_Ele1	Non-LTR Retrotransposon	An. gambiae	355	366	1.00E-101	86%
TF112352	RTE_Ele2, Jammin-2	Non-LTR Retrotransposon	An. gambiae	282	211	4.00E-55	87%

element had increased expression following heat-sock, hydrogen peroxide and sodium azide treatments. (Strand & Mcdonald, 1985). A Ty1element was also found to be increasingly expressed and mobile following adenine starvation (Todeschini et al., 2005).

The interesting possibility that stress could lead to a modulation of the piRNA pathway in order to regulate transposon activity along with our preliminary analysis of transposon transcript presence after a blood meal in *An. stephensi* midguts prompted a test as to whether heat-stress could induce mobility in the synthetic autonomous transgenic line. 3.8nanos-attP-44C-VM adult females were heat treated at 24 hr PBM for three generations beginning with the parental cross. F_1, F_2 and F_3 offspring were screened for e*CFP* or *DsRed* only in the eyes that would indicate a remobilization event. No exceptional phenotypes were seen at any generation (Table 3).

The impact of oxidative stress on Piwi gene expression in adult female tissues and embryos

To assay the impact of oxidative stress on Piwi gene transcript accumulation, 5 day-old females were offered calf-blood or calf-blood laced with paraquat dichloride hydrate at either 10 μM or 100 μM. Pairs of ovaries were dissected from the carcass at 24 hr PBM, when the blood is still being digested and 72 hr PBM when the blood is completely digested and the eggs are ready for deposition. The cage fed with 10 μM paraquat had robust survival and ovaries appeared to develop normally. A higher mortality was observed qualitatively in the cage fed with 100 μM paraquat, and during dissection it was clear that

Table 3: Phenotypes of offspring produced from heat-treated *Anopheles stephensi.*

		Temperature (^oC)		
	Eye-color	**40**	**15**	**26**
F1	**Red/Cyan**	121	341	408
	-/-	113	361	395
F2	**Red/Cyan**	551	537	678
	-/-	649	537	744
F3	**Red/Cyan**	521	563	458
	-/-	432	507	361

there were many fewer fully-developed follicles than in control mosquitoes, indicating that the former were likely under stress. qPCR analysis showed an increased abundance of Piwi gene and *hsp90* transcript abundance only at the embryo stage (Figure 14). The four transcripts are decreased in abundance at 72 hr PBM when compared to the controls. These data are consistent with what was seen with heat stress in previous experiments; it appears that stress decreases transcript abundance in the ovaries but modestly increases abundance of the Piwi gene and Hsp90 transcripts in the embryos. This is counter-intuitive, since embryos are collected 0-2 hours following oviposition and transcripts at this stage are thought to be almost entirely deposited by the follicle cells in the ovary (Pritchard & Schubiger, 1996; Zalokar, 1976). Importantly, again, the large increase in *ago3*, *piwi* and *aub* transcripts seen in the initial outlying embryo replicate was not reproduced under conditions of oxidative stress.

It is difficult to draw conclusions based on these data since there are insufficient data points that are significantly different among groups. However, the overall trend of the data do not support a hypothesis that stress induces expression of *piwi, aub* and *ago3* in *An. stephensi.* The dynamic nature of the response of an organism to temperature stress may be a confounding factor. By looking at individual replicates as depicted in Figure 10, we see that different mosquito cohorts began with different levels of *hsp90* and responded differently to heat-stress, both in timing of the response and in transcript abundance. Likely, these differences paired with the time it takes to collect most of these samples confounds the identification of the peak of transcript abundance. It also is possible that factors other than temperature, or in addition to temperature, caused the increased abundance.

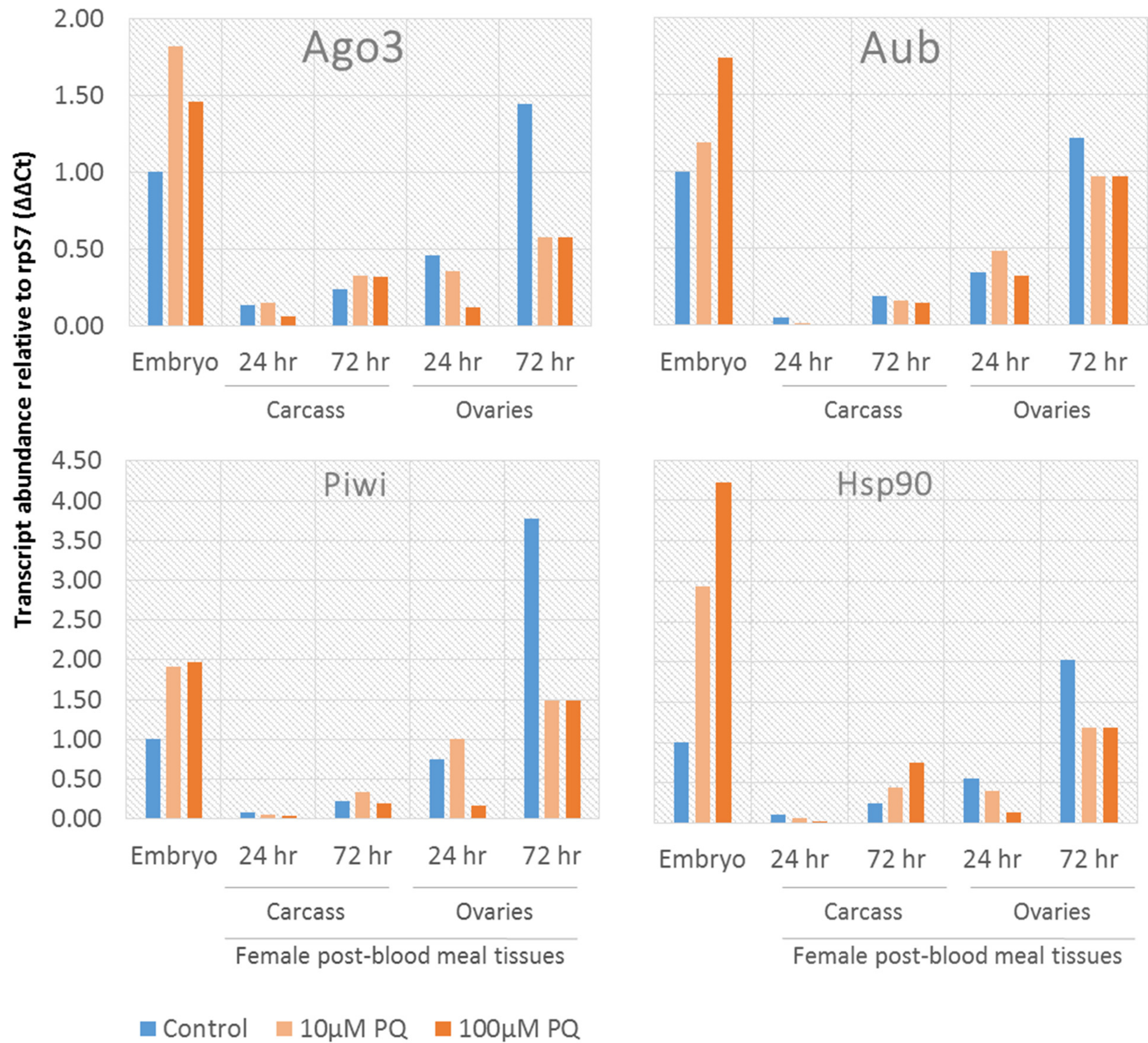


Figure 14: Piwi and *hsp90* expression following oxidative stress. Quantitative PCR analysis of adult female ovaries and carcass 24 and 72 hours after a control blood-meal or a blood-meal consisting of calf blood and 10 µM or 100 µM Paraquat. Each tissue sample was prepared from ~20 mosquitoes, each embryo sample from ~300 embryos.

Methods

All heat and cold treatments were performed at 40°C or 15°C, respectively, in large incubators (Excella E25 and Innova 4230 Refrigerated Incubator/Shaker). Entire mosquito cages were placed in the incubators with pre-warmed/cooled sugar pads, to prevent the mosquitoes from modulating body temperature by ingestion of sugar water. Collection times are with respect to the beginning of a one-hour temperature treatment, with one-hour embryos representing embryos laid during the treatment.

Total RNA extraction, cDNA synthesis and qPCR analysis were performed as described in Chapter 2.

Paraquat solutions were prepared by dissolving paraquat dichloride hydrate PESTANAL (Sigma-Aldrich) in water. Paraquat-laced blood meals were produced by adding aliquots of 2 µL or 20 µL of 100 µM paraquat solution in 2 mL of calf blood (Colorado Serum Company). Five-to-seven day-old female *An. stephensi* were offered 2 mL of either paraquat-laced or control blood meal warmed using a Hemotek membrane blood-feeding system for about 45 minutes or until most of the mosquitoes finished feeding. Mosquitoes were provided sugar pads before and after blood feeding and during all stress treatments.

Chapter 5

Perspectives and Future Directions

The experiments presented herein represent the beginning of exploring the biological interplay between the malaria vector *An. stephensi* and exogenously-integrated DNA sequences. First, we demonstrate that not only can a synthetic transposon construct be integrated into the mosquito genome, as is used routinely for mosquito transgenesis, but it can also be encoded with its own source of transposase that can mediate self-mobilization. While this is relatively rare, it is also remarkable; autonomous transposons have been synthesized that are able to mediate their own movement from a plasmid into chromosomal DNA, but have never been shown to be actively mobilized from a chromosomal insertion site to another chromosomal insertion site. This is not surprising. Most of the transposons integrated into eukaryotic genomes are inactive, either mutated so as to not contain functional mediators of transposition or post-transcriptionally-repressed by the cell (Hartl, 1997). Recently, it has been demonstrated that the cell can execute post-transcriptional repression of transposon mobilization through the piRNA pathway (Brennecke et al., 2007; Pélisson et al., 2007; Saito et al., 2006; Sarot et al., 2004; Vagin et al., 2006). Considering the low remobilization rate that we observed in the 3.8nanos-attP-44C line, and considering that the piRNA pathway has been demonstrated to act on non-autonomous exogenously-derived DNA (Le Thomas et al., 2014; Olovnikov et al., 2013), it will be important to understand the interaction that mosquitoes have with exogenously-introduced DNA in population replacement efforts. We demonstrate that the piRNA pathway in *An. stephensi* is likely one manifestation of this interaction. This mosquito's DNA encodes the three major players of the piRNA pathway, *piwi, aub* and *ago3*, and these

genes are expressed in the germline tissue following a blood meal, which is the appropriate expression profile for repression of heritable transposition. Further experiments were performed to test the hypothesis that the piRNA pathway is a link between stress and transposon mobilization, more specifically that the Piwi genes are upregulated by stress. These experiments led us to conclude that, under the conditions tested here, stress induction does not induce Piwi gene expression in *An. stephensi*.

In order to test directly the impact of the piRNA pathway on transposon control and identify other roles that the components of the pathway play in the mosquito, efforts are underway to generate tractable mutants of *piwi, aub* and *ago3.* Until very recently, reverse genetics has been nearly impossible in mosquitoes; best attempts made at inhibiting gene function have been through RNA interference mediated by injection of small RNAs or long double-stranded RNAs to be used by the siRNA pathway to degrade endogenous transcript. However, this technology is limited because it appears that small RNAs or dsRNA injection is completely ineffective in the perturbation of transcript levels in some tissues, specifically the ovaries; to my knowledge there are no reports of even moderate knock-down of ovary-specific genes. Some nuclease-based technologies including zinc-finger nucleases have recently been successfully used for targeting specific genes for mutations (reviewed in Gilles & Averof, 2014) since the zinc-finger domains can be programmed to identify specific regions of DNA and these domains can be linked to the corresponding nuclease, which can cleave those specific regions. Limitations of these technologies meant that work involving targeted gene mutation in mosquitoes was still relatively slow in coming.

This, however, is changing. With the advent of technologies based on a bacterial Cas9 nuclease, reverse genetics in mosquitoes is moving rapidly forward. The Cas9 nuclease in bacteria is targeted to specific nucleotide regions by a set of small RNAs, one that contains sequence to target the specific DNA region, and one to scaffold the specific RNA to the Cas9 protein. Jinek *et al.* (2012) reported the use of a single guide RNA (sgRNA) that contains both the scaffolding region and the specific target region in directed DNA cleavage (Jinek et al., 2012). It has been found since that this easily programmable system can be used to generate targeting mutations in many organisms.

To date, there is no report of using Cas9-mediated gene targeting for directed mutation in *An. Stephensi*, although it has been reported in print for *Ae. aegypti* and anecdotally in *An. gambaie.* With hopes that Cas9 could be used in *An. stephensi,* I began injections of a dual reporter transgenic mosquito line that expresses both eCFP and DsRed in the eyes with different Cas9 sources and sgRNAs against eCFP that had been used previously and successfully against GFP in humans (eGFP and eCFP are nearly identical in nucleotide sequence) (Zhang et al., 2014). I found that injecting Cas9 protein with *in vitro*-synthesized sgRNAs against eCFP results in heritable knockdown of eCFP in 12.5% of G_0 individuals. From these data we are confident that Cas9 can induce directed cleavage, but a tractable mutation requires a "knock-in" of a marker gene. To this end and to the end of directly testing the function of piRNA pathway in mosquitoes, efforts are underway to target *piwi, aub,* and *ago3* for Cas9-directed cleavage while at the same time providing a

template for homologous recombination with a fluorescent marker (Figure 15) into the open reading from of each gene.

Mutants for the piRNA pathway genes in *D. melanogaster* are homozygous sterile; if this also is true in *An. stephensi,* we expect to maintain mutated transgenic lines by outcrossing. Outcrossed mosquitoes will be collected at each generation and total RNA collected from this line will be used for RNA sequencing of mRNA and small RNAs. Consistent with a hypothesis that the Piwi genes have a role in transposon control, I expect that the piRNA profile will be changed in mutant mosquitoes. More specifically I expect to see an increased abundance in transposon transcripts and RNA intermediates that are under the control of the pathway. Considering data reviewed in Chapter 2 from other groups, I also might observe a change in the transcript levels of endogenous genes. Further, I am curious as to whether the low frequency of mobility of the synthetic autonomous construct examined in Chapter 3 is being actively repressed by this pathway. A simple series of crosses between Piwi gene mutant lines and the 3.8nanos-attP-44C-VM line may answer this question. Should we observe an increase in mobilization of the autonomous transcript, we could conclude that the piRNA pathway is repressing mobilization under normal circumstances.

These experiments could provide a valuable insight into the interactions between mosquito transposon control biology and exogenous DNA sequences in transgenic mosquitoes. As genetically-modified mosquitoes are becoming an increasingly viable

prospect for disease control, a deep understanding of this interplay allows us to proceed intelligently with design of genetic strains to be used and provides us with a groundwork to understand the activity of transgenes in wild populations.

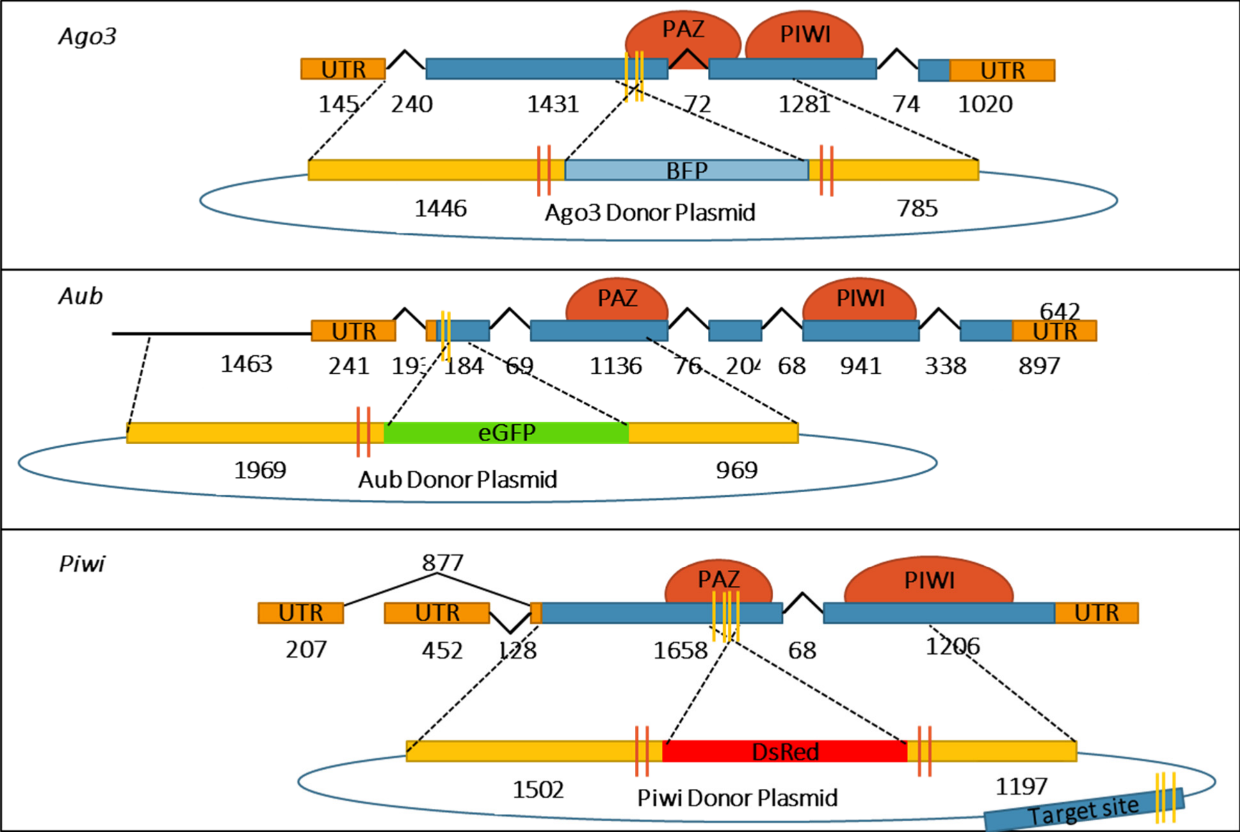


Figure 15: Schematic representation of donor plasmid design for integration of fluorescent markers into Piwi gene open reading frames. Target sites on each gene are represented by yellow lines. Corresponding sites are mutated on each donor plasmid and are represented by red lines. Regions of homology are yellow blocks.

References

Adelman, Z. N., Jasinskiene, N., Onal, S., Juhn, J., Ashikyan, A., Salampessy, M., … James, A. A. (2007). nanos gene control DNA mediates developmentally regulated transposition in the yellow fever mosquito Aedes aegypti. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(24), 9970–5. doi:10.1073/pnas.0701515104

Aguiar, E. R. G. R., Olmo, R. P., Paro, S., Ferreira, F. V., de Faria, I. J. D. S., Todjro, Y. M. H., … Marques, J. T. (2015). Sequence-independent characterization of viruses based on the pattern of viral small RNAs produced by the host. *Nucleic Acids Research*, 1–16. doi:10.1093/nar/gkv587

Akbari, O. S., Antoshechkin, I., Amrhein, H., Williams, B., Diloreto, R., Sandler, J., & Hay, B. A. (2013). The Developmental Transcriptome of the Mosquito Aedes aegypti, an Invasive Species and Major Arbovirus Vector. *G3 (Bethesda, Md.)*, *3*(9), 1493–509. doi:10.1534/g3.113.006742

Amenya, D. A., Bonizzoni, M., Isaacs, A. T., Jasinskiene, N., Chen, H., Marinotti, O., … James, A. A. (2010). Comparative fitness assessment of Anopheles stephensi transgenic lines receptive to site-specific integration. *Insect Molecular Biology*, *19*(2), 263–9. doi:10.1111/j.1365-2583.2009.00986.x

Antinori, S., Galimberti, L., Milazzo, L., & Corbellino, M. (2013). Plasmodium knowlesi: the emerging zoonotic malaria parasite. *Acta Tropica*, *125*(2), 191–201. doi:10.1016/j.actatropica.2012.10.008

Arensburger, P., Hice, R. H., Wright, J. a, Craig, N. L., & Atkinson, P. W. (2011). The mosquito Aedes aegypti has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs. *BMC Genomics*, *12*, 606. doi:10.1186/1471-2164-12-606

Bellini, R., Medici, A., Puggioli, A., Balestrino, F., & Carrieri, M. (n.d.). Pilot field trials with Aedes albopictus irradiated sterile males in Italian urban areas. - PubMed - NCBI. Retrieved August 4, 2015, from http://www.ncbi.nlm.nih.gov/pubmed/23540120

Benoit, J. B., Lopez-Martinez, G., Patrick, K. R., Phillips, Z. P., Krause, T. B., & Denlinger, D. L. (2011). Drinking a hot blood meal elicits a protective heat shock response in mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(19), 8026–9. doi:10.1073/pnas.1105195108

Biedler, J. K., Hu, W., Tae, H., & Tu, Z. (2012). Identification of early zygotic genes in the yellow fever mosquito Aedes aegypti and discovery of a motif involved in early zygotic genome activation. *PloS One*, *7*(3), e33933. doi:10.1371/journal.pone.0033933

Biedler, J., & Tu, Z. (2003). Non-LTR Retrotransposons in the African Malaria Mosquito, Anopheles gambiae: Unprecedented Diversity and Evidence of Recent Activity. *Molecular Biology and Evolution*, *20*(11), 1811–1825. doi:10.1093/molbev/msg189

Brennecke, J., Aravin, A. a, Stark, A., Dus, M., Kellis, M., Sachidanandam, R., & Hannon, G. J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell*, *128*(6), 1089–103. doi:10.1016/j.cell.2007.01.043

Calderaro, A., Piccolo, G., Gorrini, C., Rossi, S., Montecchini, S., Dell'Anna, M. L., … Arcangeletti, M. C. (2013). Accurate identification of the six human Plasmodium spp. causing imported malaria, including Plasmodium ovale wallikeri and Plasmodium knowlesi. *Malaria Journal*, *12*, 321. doi:10.1186/1475-2875-12-321

Calvo, E., Walter, M., Adelman, Z. N., Jimenez, A., Onal, S., Marinotti, O., & James, A. a. (2005). Nanos (nos) genes of the vector mosquitoes, Anopheles gambiae, Anopheles stephensi and Aedes aegypti. *Insect Biochemistry and Molecular Biology*, *35*(7), 789–98. doi:10.1016/j.ibmb.2005.02.007

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and mechanisms of miRNAs and siRNAs. *Cell*, *136*(4), 642–655. doi:10.1016/j.cell.2009.01.035.Origins

Catteruccia, F., Nolan, T., Loukeris, T. G., Blass, C., Savakis, C., Kafatos, F. C., & Crisanti, a. (2000). Stable germline transformation of the malaria mosquito Anopheles stephensi. *Nature*, *405*(6789), 959–62. doi:10.1038/35016096

Chen, D., Toone, W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., … Bähler, J. (2003). Global transcriptional responses of fission yeast to environmental stress. *Molecular Biology of the Cell*, *14*(1), 214–29. doi:10.1091/mbc.E02-08-0499

Chotkowski, H. L., Ciota, A. T., Jia, Y., Puig-Basagoiti, F., Kramer, L. D., Shi, P.-Y., & Glaser, R. L. (2008). West Nile virus infection of Drosophila melanogaster induces a protective RNAi response. *Virology*, *377*(1), 197–206. Retrieved from http://www.sciencedirect.com/science/article/pii/S0042682208002614

Coates, C. J., Jasinskiene, N., Miyashiro, L., & James, A. A. (1998). Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(7), 3748–51. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=19908&tool=pmcentrez&rendertype=abstract

Cohen, J. M., Smith, D. L., Cotter, C., Ward, A., Yamey, G., Sabot, O. J., & Moonen, B. (2012). Malaria resurgence: a systematic review and assessment of its causes. *Malaria Journal*, *11*, 122. doi:10.1186/1475-2875-11-122

Control:, T. malEra consultative group on vector, Alonso, P. L., Besansky, N. J., Burkot, T. R., Collins, F. H., Hemingway, J., … Zwiebel, L. (2011). A research agenda for malaria eradication: vector control. *PLoS Medicine*, *8*(1), e1000401. doi:10.1371/journal.pmed.1000401

Cotter, C., Sturrock, H. J. W., Hsiang, M. S., Liu, J., Phillips, A. A., Hwang, J., … Feachem, R. G. A. (2013). The changing epidemiology of malaria elimination: new strategies for new challenges. *Lancet*, *382*(9895), 900–11. doi:10.1016/S0140-6736(13)60310-4

Damiens, D., Vreysen, M. J. B., & Gilles, J. R. L. (2013). Anopheles arabiensis sperm production after genetic manipulation, dieldrin treatment, and irradiation. *Journal of Medical Entomology*, *50*(2), 314–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23540119

Daniels, S. B., Peterson, K. R., Strausbaugh, L. D., Kidwell, M. G., & Chovnick, A. (1990). Evidence for Horizontal Transmission of the P Transposable Element Between.

Djikeng, A., Shi, H., Tschudi, C., & Ullu, E. (2001). RNA interference in Trypanosoma brucei: Cloning of small interfering RNAs provides evidence for retroposon-derived 24-26-nucleotide RNAs. *RNA*, *7*(11), 1522–1530. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-0035160923&partnerID=tZOtx3y1

Elyazar, I. R. F., Sinka, M. E., Gething, P. W., Tarmidzi, S. N., Surya, A., Kusriastuti, R., … Bangs. (2013). The distribution and bionomics of anopheles malaria vector mosquitoes in Indonesia. *Advances in Parasitology*, *83*, 173–266. doi:10.1016/B978-0-12-407705-8.00003-3

Engels, W. R. (1997). Invasions of P elements. *Genetics*, *145*(1), 11–15.

Feng, G., Leem, Y.-E., & Levin, H. L. (2013). Transposon integration enhances expression of stress response genes. *Nucleic Acids Research*, *41*(2), 775–89. doi:10.1093/nar/gks1185

Fraser, M. J., Ciszczon, T., Elick, T., & Bauser, C. (1996). Precise excision of TTAA-specific lepidopteran transposons piggyBac (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. *Insect Molecular Biology*, *5*(2), 141–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8673264

Gantz, V., & Bier, E. (2015). The mutagenic chain reaction: A method for converting heterozygous to homozygous mutations. *Science*, (March). doi:10.1093/hmg/ddu125

Gato, R., Companioni, A., Bruzón, R. Y., Menéndez, Z., González, A., & Rodríguez, M. (2014). Release of thiotepa sterilized males into caged populations of Aedes aegypti: life table analysis. *Acta Tropica*, *132 Suppl*, S164–9. doi:10.1016/j.actatropica.2013.09.024

Gilles, A. F., & Averof, M. (2014). Functional genetics for all: engineered nucleases, CRISPR and the gene editing revolution. *EvoDevo*, *5*, 43. doi:10.1186/2041-9139-5-43

Grossman, G. L., Rafferty, C. S., Clayton, J. R., Stevens, T. K., Mukabayire, O., & Benedict, M. Q. (2001). Germline transformation of the malaria vector, Anopheles gambiae, with the piggyBac transposable element. *Insect Molecular Biology*, *10*(6), 597–604. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11903629

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., … Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in Drosophila. *Science (New York, N.Y.)*, *315*(5818), 1587–90. doi:10.1126/science.1140494

Hamilton, A. (2002). Two classes of short interfering RNA in RNA silencing. *The EMBO Journal*, *21*(17), 4671–4679. doi:10.1093/emboj/cdf464

Handler, A. M., McCombs, S. D., Fraser, M. J., & Saul, S. H. (1998). The lepidopteran transposon vector, piggyBac, mediates germ-line transformation in the Mediterranean fruit fly. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(13), 7520–7525. doi:10.1073/pnas.95.13.7520

Hardy, J. L., Houk, E. J., Kramer, L. D., & Reeves, W. C. (1983). Intrinsic Factors Affecting Vector Competence of Mosquitoes for Arboviruses. *Annual Review of Entomology*, *28*, 229–262. doi:DOI: 10.1146/annurev.en.28.010183.001305

Harris, a N., & Macdonald, P. M. (2001). Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. *Development (Cambridge, England)*, *128*(14), 2823–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11526087

Harris, C., Lwetoijera, D. W., Dongus, S., Matowo, N. S., Lorenz, L. M., Devine, G. J., & Majambere, S. (2013). Sterilising effects of pyriproxyfen on Anopheles arabiensis and its potential use in malaria control. *Parasites & Vectors*, *6*, 144. doi:10.1186/1756-3305-6-144

Hartl, D. (1997). What restricts the activity of mariner-like transposable elements? *Trends in Genetics*, *13*(5), 197–201. doi:10.1016/S0168-9525(97)01087-1

Hay, S. I., Sinka, M. E., Okara, R. M., Kabaria, C. W., Mbithi, P. M., Tago, C. C., … Godfray, H. C. J. (2010). Developing global maps of the dominant anopheles vectors of human malaria. *PLoS Medicine*, *7*(2), e1000209. doi:10.1371/journal.pmed.1000209

Hoa, N. ., Keene, K. ., Olson, K. ., & Zheng, L. (2003). Characterization of RNA interference in an Anopheles gambiae cell line. *Insect Biochemistry and Molecular Biology*, *33*(9), 949–957. doi:10.1016/S0965-1748(03)00101-2

Höck, J., & Meister, G. (2008). The Argonaute protein family. *Genome Biology*, *9*(2), 210. doi:10.1186/gb-2008-9-2-210

Holt, R. A., Subramanian, G. M., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., … Hoffman, S. L. (2002). The genome sequence of the malaria mosquito Anopheles gambiae. *Science (New York, N.Y.)*, *298*(5591), 129–49. doi:10.1126/science.1076181

Isaacs, A. T., Jasinskiene, N., Tretiakov, M., Thiery, I., Zettor, A., Bourgouin, C., & James, A. A. (2012). Transgenic Anopheles stephensi coexpressing single-chain antibodies resist Plasmodium falciparum development. *Proc Natl Acad Sci U S A*, *109*(28), E1922–30. doi:1207738109 [pii] 10.1073/pnas.1207738109

Isaacs, A. T., Li, F., Jasinskiene, N., Chen, X., Nirmala, X., Marinotti, O., … James, A. a. (2011). Engineered resistance to Plasmodium falciparum development in transgenic Anopheles stephensi. *PLoS Pathogens*, *7*(4), e1002017. doi:10.1371/journal.ppat.1002017

James, A. A. (2005). Gene drive systems in mosquitoes: rules of the road. *Trends Parasitol*, *21*(2), 64–67. doi:S1471-4922(04)00290-9 [pii] 10.1016/j.pt.2004.11.004

James, A. A., Blackmer, K., & Racioppi, J. V. (1989). A salivary gland-specific, maltase-like gene of the vector Aedes aegypti.pdf. *Gene*, *75*, 73–83.

Jayaraman, K. S. (1997). Consortium aims to revive sterile-mosquito project. *Nature*, *389*(6646), 6. doi:10.1038/37835

Jimenez, A. J. (2009). *Characterization of Candidate Genes for Applications in Genetic Control Strategies of Mosquito-Borne Diseases*. University of California, Irvine.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (New York, N.Y.)*, *337*(6096), 816–21. doi:10.1126/science.1225829

Kaminker, J., Bergman, C., Kronmiller, B., Carlson, J., Svirskas, R., Patel, S., … Celniker, S. (2002). The transposable elements of the Drosophila melanogaster euchromatin: a genomics perspective. *Genome Biology*, *3*(12), research0084.1–0084.20. doi:10.1186/gb-2002-3-12-research0084

Kidwell, M. G., Kidwell, J. F., & Sved, J. A. (1977). Hybrid Dysgenesis in Drosophila melanogaster: A Syndrome of Aberrant Traits Including Mutation, Sterility and Male Recombination. *Genetics*, *86*, 813–833.

Kidwell, M., & Ribeiro, J. (1992). Can transposable elements be used to drive disease refractoriness genes into vector populations? *Parasitology Today*. Retrieved from http://www.sciencedirect.com/science/article/pii/016947589290065A

Kiszewski, A., Mellinger, A., Spielman, A., Malaney, P., Sachs, S. E., & Sachs, J. (2004). A global index representing the stability of malaria transmission. *The American Journal of Tropical Medicine and Hygiene*, *70*(5), 486–98. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15155980

Klassen, W., & Curtis, C. F. (2005). HISTORY OF THE STERILE INSECT TECHNIQUE. In *The sterile insect technique: principles and practice in area-wide integrated pest management* (pp. 3–36).

Kurscheid, S., Lew-Tabor, A. E., Rodriguez Valle, M., Bruyeres, A. G., Doogan, V. J., Munderloh, U. G., … Bellgard, M. I. (2009). Evidence of a tick RNAi pathway by comparative genomics and reverse genetics screen of targets with known loss-of-function phenotypes in Drosophila. *BMC Molecular Biology*, *10*(1), 26. doi:10.1186/1471-2199-10-26

Le Thomas, A., Stuwe, E., Li, S., Du, J., Marinov, G., Rozhkov, N., … Aravin, A. A. (2014). Transgenerationally inherited piRNAs trigger piRNA biogenesis by changing the chromatin of piRNA clusters and inducing precursor processing. - PubMed - NCBI. *Genes*, *28*(15), 1167–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/?term=Transgenerationally+inherited+piRNAs+trigger+piRNA+biogenesis+by+changing+the+chromatin+of+piRNA+clusters+and+inducing+precursor+processing

Léger, P., Lara, E., Jagla, B., Sismeiro, O., Mansuroglu, Z., Coppée, J. Y., … Bouloy, M. (2013). Dicer-2- and Piwi-mediated RNA interference in Rift Valley fever virus-infected mosquito cells. *Journal of Virology*, *87*(3), 1631–48. doi:10.1128/JVI.02795-12

Li, C., Vagin, V. V, Lee, S., Xu, J., Ma, S., Seitz, H., … Zamore, P. D. (2009). Without Argonaute3, Aubergine-bound piRNAs collapse but Piwi-bound piRNAs persist. *Cell*, *137*(3), 509–521. doi:10.1016/j.cell.2009.04.027.Without

Llave, C. (2002). Endogenous and Silencing-Associated Small RNAs in Plants. *THE PLANT CELL ONLINE*, *14*(7), 1605–1619. doi:10.1105/tpc.003210

Luckhart, S., Lindsay, S. W., James, A. A., & Scott, T. W. (2010). Reframing critical needs in vector biology and management of vector-borne disease. *PLoS Neglected Tropical Diseases*, *4*(2), e566. doi:10.1371/journal.pntd.0000566

Lwetoijera, D. W., Harris, C., Kiware, S. S., Killeen, G. F., Dongus, S., Devine, G. J., & Majambere, S. (2014). Comprehensive sterilization of malaria vectors using pyriproxyfen: a step closer to malaria elimination. *The American Journal of Tropical Medicine and Hygiene*, *90*(5), 852–5. doi:10.4269/ajtmh.13-0550

Macias, V. M., & James, A. A. (2015). Impact of Genetic Modification of Vector Populations on the Malaria Eradication Agenda. In Z. Adelman (Ed.), *Genetic Control of Malaria and Dengue* (pp. 423–444). Elsevier.

Madakacherry, O., Lees, R. S., & Gilles, J. R. L. (2014). Aedes albopictus (Skuse) males in laboratory and semi-field cages: Release ratios and mating competitiveness. *Acta Tropica*, *132*, S124–S129. doi:10.1016/j.actatropica.2013.11.020

Maïga, H., Damiens, D., Niang, A., Sawadogo, S. P., Fatherhaman, O., Lees, R. S., … Gilles, J. R. (2014). Mating competitiveness of sterile male Anopheles coluzzii in large cages. *Malaria Journal*, *13*(1), 460. doi:10.1186/1475-2875-13-460

Marinotti, O., Cerqueira, G. C., de Almeida, L. G. P., Ferro, M. I. T., Loreto, E. L. da S., Zaha, A., … de Vasconcelos, A. T. R. (2013). The genome of Anopheles darlingi, the main neotropical malaria vector. *Nucleic Acids Research*, *41*(15), 7387–400. doi:10.1093/nar/gkt484

Marinotti, O., Nguyen, Q. K., Calvo, E., James, A. A., & Ribeiro, J. M. C. (2005). Microarray analysis of genes showing variable expression following a blood meal in Anopheles gambiae. *Insect Molecular Biology*, *14*(4), 365–73. doi:10.1111/j.1365-2583.2005.00567.x

McClintock, B. (1987). *The Discovery and Characterization of Tranposable Elements: The Collected Papers of Barbara McClintock*. New York: Garland Publishing.

Mette, M. F., van der Winden, J., Matzke, M., & Matzke, A. J. M. (2002). Short RNAs can identify new candidate transposable element families in Arabidopsis. *Plant Physiology*, *130*(1), 6–9. doi:10.1104/pp.007047

Morazzani, E. M., Wiley, M. R., Murreddu, M. G., Adelman, Z. N., & Myles, K. M. (2012). Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the mosquito soma. *PLoS Pathogens*, *8*(1), e1002470. doi:10.1371/journal.ppat.1002470

Ndo, C., Yamada, H., Damiens, D. D., N'do, S., Seballos, G., & Gilles, J. R. L. (2014). X-ray sterilization of the An. arabiensis genetic sexing strain "ANO IPCL1" at pupal and adult stages. *Acta Tropica*, *131*, 124–8. doi:10.1016/j.actatropica.2013.11.027

Nene, V., Wortman, J. R., Lawson, D., Haas, B., Kodira, C., Tu, Z. J., … Severson, D. W. (2007). Genome sequence of Aedes aegypti, a major arbovirus vector. *Science (New York, N.Y.)*, *316*(5832), 1718–23. doi:10.1126/science.1138878

Nimmo, D. D., Alphey, L., Meredith, J. M., & Eggleston, P. (2006). High efficiency site-specific genetic engineering of the mosquito genome. *Insect Molecular Biology*, *15*(2), 129–136. doi:10.1111/j.1365-2583.2006.00615.x

Oh, New Delhi; oh, Geneva. (editorial). (1975). *Nature*, *256*, 355–357. doi:doi:10.1038/256355a0

Oliva, C. F., Maier, M. J., Gilles, J., Jacquet, M., Lemperiere, G., Quilici, S., … Boyer, S. (2013). Effects of irradiation, presence of females, and sugar supply on the longevity of sterile

males Aedes albopictus (Skuse) under semi-field conditions on Reunion Island. *Acta Tropica*, *125*(3), 287–93. doi:10.1016/j.actatropica.2012.11.010

Oliva, C. F., Vreysen, M. J. B., Dupé, S., Lees, R. S., Gilles, J. R. L., Gouagna, L.-C., & Chhem, R. (2014). Current status and future challenges for controlling malaria with the sterile insect technique: technical and social perspectives. *Acta Tropica*, *132 Suppl*, S130–9. doi:10.1016/j.actatropica.2013.11.019

Olovnikov, I., Ryazansky, S., Shpiz, S., Lavrov, S., Abramov, Y., Vaury, C., … Kalmykova, A. (2013). De novo piRNA cluster formation in the Drosophila germ line triggered by transgenes containing a transcribed transposon fragment. *Nucleic Acids Research*, 1–12. doi:10.1093/nar/gkt310

Pecinka, A., & Mittelsten Scheid, O. (2012). Stress-induced chromatin changes: A critical view on their heritability. *Plant and Cell Physiology*, *53*(5), 801–808. doi:10.1093/pcp/pcs044

Pélisson, A., Sarot, E., Payen-Groschêne, G., & Bucheton, A. (2007). A novel repeat-associated small interfering RNA-mediated silencing pathway downregulates complementary sense gypsy transcripts in somatic cells of the Drosophila ovary. *Journal of Virology*, *81*(4), 1951–60. doi:10.1128/JVI.01980-06

Center for Disease Control and Prevention: Plasmodium vivax malaria--San Diego County, California, 1986. (1986). *MMWR. Morbidity and Mortality Weekly Report*, *35*(43), 679–81.

Potter, C. J., & Luo, L. (2010). Splinkerette PCR for mapping transposable elements in Drosophila. *PloS One*, *5*(4), e10168. doi:10.1371/journal.pone.0010168

Poulsen, C., Vaucheret, H., & Brodersen, P. (2013). Lessons on RNA silencing mechanisms in plants from eukaryotic argonaute structures. *The Plant Cell*, *25*(1), 22–37. doi:10.1105/tpc.112.105643

Pritchard, D. K., & Schubiger, G. (1996). Activation of transcription in Drosophila embryos is a gradual process mediated by the nucleocytoplasmic ratio. *Genes & Development*, *10*(9), 1131–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8654928

Rasgon, J. L., & Gould, F. (2005). Transposable element insertion location bias and the dynamics of gene drive in mosquito populations. *Insect Molecular Biology*, *14*(5), 493–500. doi:10.1111/j.1365-2583.2005.00580.x

Ratner, V. A., Zabanov, S. A., Kolesnikova, O. V, & Vasilyeva, L. A. (1992). Induction of the mobile genetic element Dm-412 transpositions in the Drosophila genome by heat shock treatment. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(12), 5650–4. Retrieved from

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=49350&tool=pmcentrez&rendertype=abstract

Reinhart, B. J., & Bartel, D. P. (2002). Small RNAs correspond to centromere heterochromatic repeats. *Science (New York, N.Y.)*, *297*(5588), 1831. doi:10.1126/science.1077183

Ribeiro, J. M., & Kidwell, M. G. (1994). Transposable elements as population drive mechanisms: specification of critical parameter values. *Journal of Medical Entomology*, *31*(1), 10–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8158612

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 539–42. doi:10.1093/sysbio/sys029

Saito, K., Nishida, K. M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., … Siomi, M. C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. *Genes & Development*, *20*(16), 2214–22. doi:10.1101/gad.1454806

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* (Second.). New York: Cold Spring Harbor Laboratry Press.

Sarot, E., Payen-Groschêne, G., Bucheton, A., & Pélisson, A. (2004). Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the Drosophila melanogaster flamenco gene. *Genetics*, *166*(3), 1313–21. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1470774&tool=pmcentrez&rendertype=abstract

Schnettler, E., Donald, C. L., Human, S., Watson, M., Siu, R. W. C., McFarlane, M., … Fragkoudis, R. (2013). Knockdown of piRNA pathway proteins results in enhanced Semliki Forest virus production in mosquito cells. *The Journal of General Virology*, *94*(Pt 7), 1680–9. doi:10.1099/vir.0.053850-0

Sehgal, A., Lee, C.-Y. S., & Espenshade, P. J. (2007). SREBP controls oxygen-dependent mobilization of retrotransposons in fission yeast. *PLoS Genetics*, *3*(8), e131. doi:10.1371/journal.pgen.0030131

Sheffield, P. E., & Landrigan, P. J. (2011). Global climate change and children's health: threats and strategies for prevention. *Environmental Health Perspectives*, *119*(3), 291–8. doi:10.1289/ehp.1002233

Smith, C. D., Shu, S., Mungall, C. J., & Karpen, G. H. (2007). The Release 5.1 annotation of Drosophila melanogaster heterochromatin. *Science (New York, N.Y.)*, *316*(5831), 1586–91. doi:10.1126/science.1139815

Smith, D. L., Battle, K. E., Hay, S. I., Barker, C. M., Scott, T. W., & McKenzie, F. E. (2012). Ross, macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens. *PLoS Pathogens*, *8*(4), e1002588. doi:10.1371/journal.ppat.1002588

Sokhna, C., Ndiath, M. O., & Rogier, C. (2013). The changes in mosquito vector behaviour and the emerging resistance to insecticides will challenge the decline of malaria. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *19*(10), 902–7. doi:10.1111/1469-0691.12314

Spielman, A., Kitron, U., & Pollack, R. J. (1993). Time limitation and the role of research in the worldwide attempt to eradicate malaria. *Journal of Medical Entomology*, *30*(1), 6–19. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8094462

Spradling, A. C., & Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. *Science (New York, N.Y.)*, *218*(4570), 341–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6289435

Strand, D. J., & Mcdonald, J. F. (1985). Copia is transcriptionally responsive to enviornmental stress. *Nucleic Acids Research*, *13*(12), 4401–4410.

Su, J., Zhu, Z., Wang, Y., & Jang, S. (2009). Isolation and characterization of Argonaute 2: A key gene of the RNA interference pathway in the rare minnow, Gobiocypris rarus. *Fish & Shellfish Immunology*, *26*(1), 164–170. Retrieved from http://www.sciencedirect.com/science/article/pii/S1050464808002325

Tatarsky, A., Aboobakar, S., Cohen, J. M., Gopee, N., Bheecarry, A., Moonasar, D., … Sabot, O. (2011). Preventing the reintroduction of malaria in Mauritius: a programmatic and financial assessment. *PloS One*, *6*(9), e23832. doi:10.1371/journal.pone.0023832

Terenius, O., Marinotti, O., Sieglaff, D., & James, A. A. (2008). Molecular genetic manipulation of vector mosquitoes. *Cell Host Microbe*, *4*(5), 417–423. doi:S1931-3128(08)00296-5 [pii] 10.1016/j.chom.2008.09.002

The malERA Consultative Group on Integration Strategies:, Alonso, P. L., Brown, G., & Tanner, M. (2011). A research agenda for malaria eradication: cross-cutting issues for eradication. *PLoS Medicine*, *8*(1), e1000404. doi:10.1371/journal.pmed.1000404

Todeschini, A.-L., Morillon, A., Springer, M., & Lesage, P. (2005). Severe adenine starvation activates Ty1 transcription and retrotransposition in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *25*(17), 7459–72. doi:10.1128/MCB.25.17.7459-7472.2005

Vagin, V. V, Sigova, A., Li, C., Seitz, H., Gvozdev, V., & Zamore, P. D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science (New York, N.Y.)*, *313*(5785), 320–4. doi:10.1126/science.1129333

Vodovar, N., Bronkhorst, A. W., van Cleef, K. W. R., Miesen, P., Blanc, H., van Rij, R. P., & Saleh, M.-C. (2012). Arbovirus-derived piRNAs exhibit a ping-pong signature in mosquito cells. *PloS One*, *7*(1), e30861. doi:10.1371/journal.pone.0030861

White, N. J., Pukrittayakamee, S., Hien, T. T., Faiz, M. A., Mokuolu, O. A., & Dondorp, A. M. (2014). Malaria. *Lancet*, *383*(9918), 723–35. doi:10.1016/S0140-6736(13)60024-0

WHO. (1991). Prospects for Malaria Control by Genetic Manipulation of Its Vectors.

WHO. (2013). *World Malaria Report*. World Health Organization. Retrieved from http://www.who.int/malaria/publications/world_malaria_report_2013/report/en/

WHO. (2014). WHO procedures for certification of malaria elimination. *Weekly Epidemiological Record*, *89*(29), 321–325. Retrieved from http://www.pubfacts.com/detail/25045772/WHO-procedures-for-certification-of-malaria-elimination.

Yamada, H., Vreysen, M. J. B., Bourtzis, K., Tschirk, W., Chadee, D. D., & Gilles, J. R. L. (2015). The Anopheles arabiensis genetic sexing strain ANO IPCL1 and its application potential for the sterile insect technique in integrated vector management programmes. *Acta Tropica*, *142*, 138–144. doi:10.1016/j.actatropica.2014.11.013

Yamada, H., Vreysen, M. J. B., Gilles, J. R. L., Munhenga, G., & Damiens, D. D. (2014). The effects of genetic manipulation, dieldrin treatment and irradiation on the mating competitiveness of male Anopheles arabiensis in field cages. *Malaria Journal*, *13*, 318. doi:10.1186/1475-2875-13-318

Zalokar, M. (1976). Autoradiographic study of protein and RNA formation during early development of Drosophila eggs. *Developmental Biology*, *49*(2), 425–437. Retrieved from http://www.sciencedirect.com/science/article/pii/0012160676901858

Zhang, Y., Ge, X., Yang, F., Zhang, L., Zheng, J., Tan, X., … Gu, F. (2014). Comparison of non-canonical PAMs for CRISPR/Cas9-mediated DNA cleavage in human cells. *Scientific Reports*, *4*, 5405. doi:10.1038/srep05405

Zheng, Y. (2013). Phylogenetic analysis of the Argonaute protein family in platyhelminths. *Molecular Phylogenetics and Evolution*, *66*(3), 1050–1054. Retrieved from http://www.sciencedirect.com/science/article/pii/S1055790312004630

Zhou, X., Liao, Z., Jia, Q., Cheng, L., & Li, F. (2007). Identification and characterization of Piwi subfamily in insects. *Biochemical and Biophysical Research Communications*, *362*(1), 126–31. doi:10.1016/j.bbrc.2007.07.179

Zimmerman, J. L., Petri, W., & Meselson, M. (1983). Accumulation of a specific subset of D. melanogaster heat shock mRNAs in normal development without heat shock. *Cell*, *32*(4), 1161–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6404558

Appendix 1; p-values from statistical analyses.

Table A1-1: p-values from statistical analysis using two-sided t-tests of tissue and time point expression as measured by qPCR. Biorad software (Version 3.0) was used for statistical analysis with the default two-sided t-test with a p-value threshold of 0.05 set for significance. p-values less than 0.05 and 0.001 are highlighted in light and dark orange, respectively. N/A indicates little or no amplification of the target gene in one of the compared samples in technical and biological replicates, which prohibited performance of the test. This occurred for piwi amplified from male samples and female carcass samples.

	Tissue		Embryo	Larvae	Pupae	Male	Female Carcass PBM					Female Ovaries PBM				
Gene		Timepoint					0 hr	2 hr	24 hr	48 hr	72 hr	0 hr	2 hr	24 hr	48 hr	72 hr
Ago3	Embryo															
	Larvae		0.0031													
	Pupae		0.5915	0.0988												
	Male		0.1256	0.0561	0.1274											
	Female Carcass PBM	0 hr	0.0006	0.1580	0.0381	0.0472										
		2 hr	0.0034	0.7066	0.0954	0.0691	0.3396									
		24 hr	0.0003	0.0487	0.0270	0.0448	0.6071	0.1411								
		48 hr	0.9310	0.0403	0.5937	0.0411	0.0156	0.0416	0.0114							
		72 hr	0.0045	0.9389	0.1117	0.0568	0.1952	0.6896	0.0810	0.0441						
	Female Ovaries PBM	0 hr	0.0130	0.8574	0.1559	0.0589	0.2992	0.6880	0.1860	0.0566	0.9061					
		2 hr	0.8558	0.0891	0.8016	0.1222	0.0399	0.0897	0.0303	0.8047	0.0977	0.1263				
		24 hr	0.7325	0.0872	0.5364	0.1672	0.0494	0.0931	0.0409	0.7608	0.0924	0.1088	0.6752			
		48 hr	0.0023	0.0008	0.0031	0.1450	0.0006	0.0014	0.0006	0.0001	0.0008	0.0009	0.0022	0.0034		
		72 hr	0.0000	0.0000	0.0000	0.9517	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0011	0.0670	

Table A1-1 continued

	Tissue		Embryo	Larvae	Pupae	Male	Female Carcass PBM					Female Ovaries PBM				
Gene		Timepoint					0 hr	2 hr	24 hr	48 hr	72 hr	0 hr	2 hr	24 hr	48 hr	72 hr
Aub	Embryo															
	Larvae		0.0003													
	Pupae		0.0002	0.6728												
	Male		0.0034	0.6012	0.4092											
	Female Carcass PBM	0 hr	0.0000	0.1562	0.3397	0.1368										
		2 hr	0.0001	0.3059	0.5855	0.2340	0.5657									
		24 hr	0.0000	0.0165	0.0457	0.0419	0.1027	0.0146								
		48 hr	0.0000	0.6404	0.3642	0.8096	0.0647	0.1524	0.0081							
		72 hr	0.0000	0.0231	0.0670	0.0458	0.2126	0.0415	0.4714	0.0102						
	Female Ovaries PBM	0 hr	0.8853	0.1226	0.1000	0.1840	0.0646	0.0952	0.0536	0.0450	0.0456					
		2 hr	0.5716	0.0537	0.0445	0.0790	0.0301	0.0469	0.0283	0.0098	0.0222	0.5828				
		24 hr	0.4040	0.0351	0.0293	0.0518	0.0199	0.0321	0.0196	0.0045	0.0148	0.4399	0.8225			
		48 hr	0.0067	0.0004	0.0003	0.0006	0.0002	0.0006	0.0003	0.0000	0.0002	0.0088	0.0313	0.0512		
		72 hr	0.0030	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1141	0.4935	0.7207	0.0549	
Piwi		Embryo														
		Larvae	0.0000													
		Pupae	0.0000	0.0818												
		Male	N/A	N/A	N/A											
	Female Carcass PBM	0 hr	0.0000	0.0304	0.0003	N/A										
		2 hr	N/A	N/A	N/A	N/A	N/A									
		24 hr	0.0000	0.0110	0.0001	N/A	0.2045	N/A								
		48 hr	N/A	N/A	N/A	N/A	N/A	N/A	N/A							
		72 hr	0.0000	0.5909	0.0222	N/A	0.0671	N/A	0.0253	N/A						
	Female Ovaries PBM	0 hr	0.0000	0.7588	0.0885	N/A	0.0023	N/A	0.0005	N/A	0.3449					
		2 hr	0.0009	0.0113	0.0769	N/A	0.0023	N/A	0.0009	N/A	0.0057	0.0126				
		24 hr	0.0000	0.0110	0.0001	N/A	0.2045	N/A	N/A	N/A	0.0253	0.0005	0.0009			
		48 hr	0.0514	0.0005	0.0007	N/A	0.0012	N/A	0.0005	N/A	0.0004	0.0005	0.0017	0.0011		
		72 hr	0.0000	0.0000	0.0000	N/A	0.0000	N/A	0.0000	N/A	0.0000	0.0000	0.0000	0.0000	0.8410	

Table A1-2: P-values from statistical analysis of qPCR amplification of *hsp90* in *An. stephensi* tissues and post-blood meal time points. Biorad software (Version 3.0) was used for statistical analysis with the default two-sided t-test with a p-value threshold of 0.05 set for significance using a two-sided t-test. P-values less than 0.05 and .0001 are highlighted in light and dark orange respectively.

Tissue		Embryo	Larvae	Pupae	Male	Female carcass post-blood meal				Female ovaries post-blood meal			
	Timepoint					O hr	2 hr	24 hr Carcass	48 hr Carcass	0 hr Ovaries	2 hr Ovaries	24 hr Ovaries	48 hr Ovaries
Embryo													
Larvae		0.013219											
Pupae		0.070928	0.034446										
Male		0.05419	0.011244	0.165363									
Carcass	O hr	0.01098	0.051499	0.019418	0.000001								
	2 hr C	0.341219	0.002258	0.114498	0.01988	0.001483							
	24 hr C	0.31818	0.122465	0.483099	0.264881	0.102301	0.778246						
	48 hr C	0.021749	0.214383	0.153986	0.803686	0.07933	0.00824	0.191357					
Ovaries	SF O	0.013101	0.011892	0.01461	0.00053	0.065033	0.001682	0.099111	0.033017				
	2 hr O	0.459213	0.000106	0.001838	0.001691	0.000082	0.032979	0.059385	0.000237	0.000141			
	24 hr O	0.065121	0.029774	0.939917	0.157852	0.01587	0.09564	0.457656	0.154944	0.011618	0.001525		
	48 hr O	0.154918	0.022139	0.038042	0.067131	0.020823	0.072241	0.067543	0.025828	0.027173	0.240484	0.037049	

Table A1- 3: p-values from statistical analysis using a two-sided t-test of qPCR amplification of Piwi genes and *hsp90* in *An. stephensi* tissues from heat shock- and control-treated female tissues. Biorad software (Version 3.0) was used for statistical analysis with the default two-sided t-test with a p-value threshold of 0.05 set for significance. p-values less than 0.05 and .0001 are highlighted in light and dark orange respectively**.** N/A indicates little or no amplification of the target gene in one of the compared samples in technical and biological replicates, which prohibited performance of the test.

	Treatment Group			Heat-stressed				Control			
		Time PBM		24 hr		72 hr		24 hr		72 hr	
Gene			Tissue	Ovaries	Carcass	Ovaries	Carcass	Ovaries	Carcass	Ovaries	Carcass
Ago3	Heat-stressed	24 hr	Ovaries								
			Carcass	0.294050							
		72 hr	Ovaries	0.000140	0.000264						
			Carcass	0.137554	0.282939	0.008737					
	Control	24 hr	Ovaries	0.099673	0.412408	0.000579	0.525284				
			Carcass	0.611902	0.094476	0.000094	0.094992	0.034227			
		72 hr	Ovaries	0.002241	0.004626	0.204160	0.082463	0.010517	0.001513		
			Carcass	0.033880	0.053853	0.275285	0.235563	0.085793	0.027772	0.897665	
Aub	Heat-stressed	24 hr	Ovaries								
			Carcass	N/A							
		72 hr	Ovaries	0.000942	N/A						
			Carcass	0.121040	N/A	0.082763					
	Control	24 hr	Ovaries	0.680903	N/A	0.002383	0.055437				
			Carcass	0.007065	N/A	0.000139	0.136413	0.030651			
		72 hr	Ovaries	0.000010	N/A	0.762331	0.000000	0.000193	0.000000		
			Carcass	0.020633	N/A	0.000181	N/A	0.055437	0.136413	0	
Hsp90	Heat-stressed	24 hr	Ovaries								
			Carcass	0.000001							
		72 hr	Ovaries	0.278896	0.000006						
			Carcass	0.012633	0.606715	0.022919					
	Control	24 hr	Ovaries	0.003433	0.000000	0.001709	0.004002				
			Carcass	0.002145	0.000000	0.001230	0.003727	0.119155			
		72 hr	Ovaries	0.927490	0.000003	0.287093	0.022369	0.000085	0.000040		
			Carcass	0.991441	0.000001	0.265765	0.012507	0.001644	0.000976	0.930943	
Piwi	Heat-stressed	24 hr	Ovaries								
			Carcass	0.003502							
		72 hr	Ovaries	0.000001	0.000000						
			Carcass	0.845096	0.010533	0.000001					
	Control	24 hr	Ovaries	0.003655	0.001101	0.000684	0.004324				
			Carcass	0.303048	0.000732	0.000003	0.314035	0.005182			
		72 hr	Ovaries	0.001065	0.000615	0.366146	0.001158	0.055317	0.002348		
			Carcass	0.041604	0.017546	0.002496	0.045930	0.777181	0.049401	0.080142	

Table A1-4: p-values from statistical analysis using a two-sided t-test of qPCR amplification of Piwi gene products and *hsp90* in *An. stephensi* tissues from heat shock- and control-treated whole females. Biorad software (Version 3.0) was used for statistical analysis with the default two-sided t-test comparing stressed sample means to 27ºC sample mean at the same time point. with a p-value threshold of 0.05 set for significance. p-values less than 0.05 and .0001 are highlighted in light and dark orange respectively.

Temp	Time Post-Stress	Gene			
		Ago3	Aub	Piwi	Hsp90
15ºC	1	0.004956	0.003753	0.127137	0.611493
	2	0.166022	0.005622	0.339070	0.236848
	4	0.659854	0.68534	0.267205	0.003299
	6	0.031064	0.002673	0.042465	0.201799
	24	0.193414	0.118619	0.097683	0.883808
40ºC	1	0.088316	0.053240	0.055013	0.017762
	2	0.598030	0.061032	0.110775	0.000000
	4	0.069798	0.06509	0.002421	0
	6	0.000841	0.111519	0.056882	0.028476
	24	0.925578	0.061492	0.764881	0.032934

Appendix 2: Primers

Primers	5'-3' Forward	5'-3' Reverse
5' RACE		
Ago3	CGCGCGCACACTCGCCAACG	CGGCCACCCCGTCGCGGAAT
Ago3 Rev 2		TGCGGGTGCGCAGCGCCTGA
Ago3 Rev 2		GCTGCGGCACGATGCGGGCCTGT
Aub	TGGCACGCGGCTTCAGGGGGA	CGGGCTGGGACACGCCGGGA
Aub Rev 1		CGGGCTGGGACACGCCGGGACA
Aub Rev 2		TGCGCCATGCCGGGCGACGCT
Piwi	CCGCCCGGTCGTGGTAATCCGATGC	ATGACGGGGCCCTGCGTGCGG
Piwi Rev 1		TGCGCCGGTATCGCCGCCTCCGT
Piwi Rev 2		GCTGGGGCGCGGCAGAATGCGGT
3' RACE		
Ago3	TACTCGCGGGCCACGATCCA	CCGGCACACGAATGGTCCCC
Aub	GCCGCCCGTGGCATGCGGTT	GCCACCGCTGCGATACACACCCCG
Aub For 1	TGCAAGCTCGGCGGCGTACCGT	
Aub For 2	CGGCCGGCATCGAGCAGGAGGTGC	
Aub For 4	GCAACATGCTCGAAAAGAAACTTTACT	
Piwi	ACCGCACACGCGGGGCGAAGA	ACTGGGCGGTGAGGAAGGCCAGC
Piwi For 1	GGCGATGGGCAGCTGCGCGGTGT	
Piwi For 2	ACCGCAATCCGCCGCCCGGCAC	
Quantitative PCR		
Ago3	AGCGTGCTCGATCATACCGT	CGTCCCGCAGCACAATGTAG
Aub	CCGGGCTGACGGATTCAATG	CGATCCGGATTCAGACGGGT
Piwi	GGTTCAAATCGGTGCCGCTG	CATTCACGTACGCGTTGCCT
Hsp90		
rpS7	GGTGCACCTGGATAAGAACCA	CGGCCAGTCAGCTTCTTGTAC
Determination of cDNA sequence		
Ago3 1	CCTCTGCCAACCGGGACAGT	CACCATTCGCTCCTGCGAGA
Ago3 2	TGTTCCGGCGCGTCATGTAT	GTTGGCAACCTTGCGCTCCT
Ago3 3	ACAAGGAAACGCCGCAGGAG	GTTGGCAACCTTGCGCTCCT
Ago3 4	GGCGATCAAACGGCTGCTCT	AGCCGGAGCATTCCCTAGCC
Aub 1	GGAGGTTATCCACCGCAAGG	ACAGAAGCGGTTGCCCAGTC
Aub 2	GACCCCTGGCGGATACACTG	TACGATCGTGCCGCTGACAT
Aub 3	GGCAGCACGAAAACGATGTG	TCGACGAGACGACGATCGAG
Aub 4	GGAGCTCGATCGTCGTCTCG	ATCGCACCGAACGATTTGCT
Aub 5	AAATCGTTCGGTGCGATGGT	TCGTGCCGGACCAATTGTAG
Piwi 1A	GGCAACACAGCTTGCGAAGA	GCTTGTGGGTTTGCATCTGCT
Piwi 1B	GCTGCTGGGTCGCATTCTTT	GCTTGTGGGTTTGCATCTGCT
Piwi 2	GCAGCAGATGCAAACCCACA	GGATCAGTGCCTGCATGACG
Piwi 3	TTGAGCCAACGGTGGAGGAT	GACGAACCGTCCTTCGTGCT
Piwi 4	CAGGTCCTCGGTGCACAGGT	AGCGGCACCGATTTGAACAT
Piwi 5	GCACCGGAACCAGATGTTCA	TGCTTCGACGCGTACATGGT
Piwi 6	CGAAGCAGACCGAACCGAAG	GTCCGCCGTGAGACCACTCT
Probes for southern blots		
eCFP	CTTGTACAGCTCGTCCATGC	ACGTAAACGGCCACAAGTTC
DsRed	CCACCAATGGTGCGCTCCTCC	ACGTACACCTTGGAGCCGTA

Appendix 2 continued

Characterization of *piggyBac* construct location		
Primer set	**Primer: 5'-3' Sequence**	**Primer: 5'-3' Sequence**
A	44C For: TTGATCCCCAATTCTGACAC	44C Rev: TCCCAGGATATTCCCATTTG
B	44C For: TTGATCCCCAATTCTGACAC	piggyBac5REV: TGACACTTACCGCATTGACA
C	CFP For: CTTGTACAGCTCGTCCATGC	CFP Rev: ACGTAAACGGCCACAAGTTC
D	Plasm2 For: GGTCGAGTAAAGCGCAAATC	piggyBac5REV: TGACACTTACCGCATTGACA
E	DsRed For: CGCTCCTCCAAGAACGTCAT	DsRed Rev: TAGTCCTCGTTGTGGGAGGT
Inverse PCR		**Reference**
piggyBac5FOR	TCTTGACCTTGCCACAGAGG	Handler 1998
piggyBac5REV	TGACACTTACCGCATTGACA	Handler 1998
piggyBac3FOR	CATTTGCCTTTCGCCTTATTTTAGA	Nimmo *et al* 2006
piggyBac3REV	AAACCTCGATATACAGACCGATAAAAACAC	Isaacs *et al* 2012